Novel biomarkers of cardiometabolic risk in population studies- With a focus on arterial stiffness and diabetes

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2020

Document Version:
Publisher's PDF, also known as Version of record

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DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY

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Iram Faqir Muhammad

LUND
UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Agardhsalen Clinical Research Centre, Jan Waldenströms gata 35,
Skånes Universitetssjukhus, Malmö, Friday 18th December 2020 at 09.00

Faculty opponent
Jonas Spaak, Associate Professor
Department of Clinical Sciences, Karolinska Institutet,
Danderyd Hospital

Organization	Document name
LUND UNIVERSITY	DOCTORAL DISSERTATION
	Date of issue 18 December 2020
Author(s): Iram Faqir Muhammad	Sponsoring organization

Title and subtitle: Novel biomarkers of cardiometabolic risk in population studies - With a focus on arterial stiffness and diabetes

Abstract

Cardiovascular disease and diabetes are the leading health problems worldwide. Early detection of individuals at high risk is, therefore, essential to reduce the burden of disease on healthcare systems, ensure better treatment outcomes and improved quality of life for these individuals. The aim of this thesis was to study the association of potential biomarkers with cardiometabolic risk in the general population.

This thesis is based on epidemiological data from two population-based cohorts to evaluate biomarkers in relation to diabetes, and vascular health outcomes, namely arterial stiffness and coronary artery calcium score (CACS). The studies were conducted in the Malmö Diet and Cancer Study- Cardiovascular Cohort (MDCS-CC) and the Swedish Cardiopulmonary Bioimage Study (SCAPIS) using longitudinal and cross-sectional design.

In Paper Ⅰ, we examined the relationship of plasma levels of five acute-phase proteins namely, ceruloplasmin, alpha-1-antitrypsin, orosomucoid, haptoglobin and C-reactive protein (CRP) and the incidence of diabetes. The results showed that orosmucoid, haptoglobin and CRP were associated with an increased risk for diabetes. However, after additional adjustment for fasting glucose levels at baseline, the association remained significant only for CRP.

In Paper Ⅱ, acute-phase proteins were investigated in relation to arterial stiffness, as determined by carotid femoral pulse wave velocity (c-f PWV). The results showed that alpha-1-antitrypsin, C3 and CRP were associated with increased arterial stiffness indicating the role of inflammation in arteriosclerosis.

In Paper Ⅲ, we assessed the incidence of diabetes in relation to arterial stiffness. We found that individuals with higher arterial stiffness were at increased risk for diabetes.

In Paper Ⅳ, HER2/ErbB2, an oncogenic marker mainly related to breast cancer, was investigated in relation to diabetes. The results showed that elevated levels of this marker were positively associated with higher risk of developing diabetes.

In Paper Ⅴ, cross-sectional association between arterial stiffness and CACS was explored in the SCAPIS cohort. It was observed that higher arterial stiffness was associated with an increased risk of higher CACS.

In summary, in this thesis we observed that elevated levels of inflammatory markers are associated with both increased risk of diabetes and higher arterial stiffness. We also observed that individuals with higher c-f PWV were at an increased risk of developing diabetes. Elevated levels of HER2/ErbB2 were also associated with an increased risk of diabetes. Higher c-f PWV was associated with risk of higher CACS, demonstrating the value of arterial stiffness assessment

Key words: Arterial stiffness, biomarkers, cohort study, diabetes mellitus, epidemiology, inflammation, oncology, plasma proteins, proteomics, risk factors, risk markers.

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language:** English
ISSN and key title **1652-8220**		**ISBN 978-91-7619-961-9**
Recipient's notes	**Number of pages** 99	Price
	Security classification	

Signature  Date 2020-11-09

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Cover photo by artist, Anas Qalamkar, edited by Hassan Ibrahim.

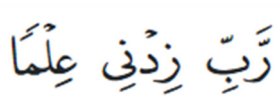
On the cover photo, the branches and leaves depict the arterial tree arising from the heart. The humming bird, known as the “bird of hope” is considered to be a symbol of diabetes. The cover was painted to represent cardiometabolic disease, and at the same time giving an element of hope.

Faculty of Medicine
Department of Clinical Sciences

ISBN **978-91-7619-961-9**
ISSN **1652-8220**

Printed in Sweden by Media-Tryck, Lund University
Lund 2020

To mom and dad, all that I am is because of you



"My Lord! Increase me in knowledge."

(Holy Quran. Surah Taha, verse 114)

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List of papers

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I. **Muhammad IF**, Borné Y, Hedblad B, Nilsson PM, Persson M, Engström G. Acute-phase proteins and incidence of diabetes: a population-based cohort study. Acta Diabetol. 2016 Dec;53(6):981-989 © Muhammad et al.

II. **Muhammad IF**, Borné Y, Östling G, Kennbäck C, Gottsäter M, Persson M, Nilsson PM, Engström G. Acute phase proteins as prospective risk markers for arterial stiffness: The Malmö Diet and Cancer cohort. PLoS One. 2017 Jul 31;12(7):e0181718 © Muhammad et al.

III. **Muhammad IF**, Borné Y, Östling G, Kennbäck C, Gottsäter M, Persson M, Nilsson PM, Engström G. Arterial Stiffness and Incidence of Diabetes: A Population-Based Cohort Study. Diabetes Care. 2017 Dec;40(12):1739-1745 © 2017 The American Diabetes Association. Diabetes Care.

IV. **Muhammad IF**, Borné Y, Bao X, Melander O, Orho-Melander M, Nilsson PM, Nilsson J, Engström G. Circulating HER2/ErbB2 Levels Are Associated With Increased Incidence of Diabetes: A Population-Based Cohort Study. Diabetes Care. 2019 Aug;42(8):1582-1588. © 2019 The American Diabetes Association. Diabetes Care.

V. **Muhammad IF**, Engvall JE, Persson M, Borné Y, Nilsson PM, Östgren CJ, Engström G. The association of arterial stiffness with coronary artery calcium score in the general population: the Swedish Cardiopulmonary Bioimage Study. *Manuscript*

Papers not included in the thesis

I. Borné Y, **Muhammad IF**, Lorés-Motta L, Hedblad B, Nilsson PM, Melander O, de Jong EK, Blom AM, den Hollander AI, Engström G. Complement C3 Associates With Incidence of Diabetes, but No Evidence of a Causal Relationship. J Clin Endocrinol Metab. 2017 Dec 1;102(12):4477-4485.

II. **Muhammad IF**, Borné Y, Melander O, Orho-Melander M, Nilsson J, Söderholm M, Engström G. FADD (Fas-Associated Protein With Death Domain), Caspase-3, and Caspase-8 and Incidence of Ischemic Stroke. Stroke. 2018 Sep;49(9):2224-2226.

III. Bao X, Borné Y, Johnson L, **Muhammad IF**, Persson M, Niu K, Engström G. Comparing the inflammatory profiles for incidence of diabetes mellitus and cardiovascular diseases: a prospective study exploring the 'common soil' hypothesis. Cardiovasc Diabetol. 2018 Jun 12;17(1):87

IV. Bao X, Borné Y, **Muhammad IF**, Nilsson J, Lind L, Melander O, Niu K, Orho-Melander M, Engström G. Growth differentiation factor 15 is positively associated with incidence of diabetes mellitus: the Malmö Diet and Cancer-Cardiovascular Cohort. Diabetologia. 2019 Jan;62(1):78-86.

V. Guo QH, **Muhammad IF**, Borné Y, Sheng CS, Persson M, Wang JG, Engström G, Li Y, Nilsson PM. Difference in the risk profiles of carotid-femoral pulse wave velocity: results from two community-based studies in China and Sweden. J Hum Hypertens. 2020 Mar;34(3):207-213

VI. Bao X, Borné Y, **Muhammad IF**, Schulz CA, Persson M, Orho-Melander M, Niu K, Christensson A, Engström G. Complement C3 and incident hospitalization due to chronic kidney disease: a population-based cohort study. BMC Nephrol. 2019 Feb 21;20(1):61

Abstract

Cardiovascular disease and diabetes are the leading health problems worldwide. Early detection of individuals at high risk is, therefore, essential to reduce the burden of disease on healthcare systems, ensure better treatment outcomes and improved quality of life for these individuals. The aim of this thesis was to study the association of potential biomarkers with cardiometabolic risk in the general population.

This thesis is based on epidemiological data from two population-based cohorts to evaluate biomarkers in relation to diabetes, and vascular health outcomes, namely arterial stiffness and coronary artery calcium score (CACS). The studies were conducted in the Malmö Diet and Cancer Study- Cardiovascular Cohort (MDCS-CC) and the Swedish Cardiopulmonary Bioimage Study (SCAPIS) using longitudinal and cross-sectional design.

In **Paper I**, we examined the relationship of plasma levels of five acute-phase proteins namely, ceruloplasmin, alpha-1-antitrypsin, orosomucoid, haptoglobin and C-reactive protein (CRP) and the incidence of diabetes. The results showed that orosmucoid, haptoglobin and CRP were associated with an increased risk for diabetes. However, after additional adjustment for fasting glucose levels at baseline, the association remained significant only for CRP.

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In **Paper III**, we assessed the incidence of diabetes in relation to arterial stiffness. We found that individuals with higher arterial stiffness were at an increased risk for diabetes.

In **Paper IV**, HER2/ErbB2, an oncogenic marker mainly related to breast cancer, was investigated in relation to diabetes. The results showed that elevated levels of this marker were positively associated with higher risk of developing diabetes.

In **Paper V**, cross-sectional association between arterial stiffness and CACS was explored in the SCAPIS cohort. It was observed that higher arterial stiffness was associated with an increased risk of higher CACS.

In summary, in this thesis we observed that elevated levels of inflammatory markers are associated with both increased risk of diabetes and higher arterial stiffness. We also observed that individuals with higher c-f PWV were at an increased risk of developing diabetes. Elevated levels of HER2/ErbB2 were also associated with an increased risk of

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Keywords: Arterial stiffness, biomarkers, cohort study, diabetes mellitus, epidemiology, inflammation, oncology, plasma proteins, proteomics, risk factors, risk markers.

Abbreviations

MDCS	Malmö Diet and Cancer study
MDCS-CC	Malmö Diet and Cancer study-Cardiovascular Cohort
IMT	Intima Media thickness
b-a PWV	Brachial-ankle pulse wave velocity
BMI	Body Mass Index
BP	Blood pressure
c-f PWV	Carotid femoral pulse wave velocity
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease
C3	Complement component 3
FPG	Fasting plasma glucose
HDL	High-density lipoprotein
HER2	Human epidermal growth factor receptor 2
HR	Hazard ratio
LDL	Low-density lipoprotein
LV	Left ventricle
MAP	Mean arterial pressure
OGTT	Oral glucose tolerance test
OR	Odds ratio
PWV	Pulse wave velocity
SCAPIS	Swedish Cardiopulmonary Bioimage Study
VSMC	Vascular smooth muscle cell
WHO	Word Health Organization

Introduction

"The work of epidemiology is related to unanswered questions, but also to unquestioned answers."

-Patricia Buffler, University of California, Epidemiologist

Epidemiology can be described as "the study of the distribution and determinants of health-related states or events in specified populations, and the application of this study to the control of health problems"(1). The science of epidemiology is an old discipline, and its roots can be traced back to as early as 400 B.C. The Greek physician Hippocrates is considered to be the first epidemiologist as he sought a logical explanation for disease occurrence. In his treatise "On Airs, Waters, and Places", Hippocrates attempted to explain the role of environmental factors in relation to disease development (2).

The evolution of epidemiology has seen important works carried out since then. John Gaunt was the first to summarize patterns of birth, death, and disease occurrence (3). William Farr further developed on the works of Gaunt and highlighted the importance of analysing death statistics. Later on, John Snow laid down the ground work for descriptive epidemiology with his work during the cholera outbreak in London in 1853-1854. Snow used geographic data and analysis to hypothesize, investigate and then prove the connection between water pumps and cholera (4). From a public health point of view, Geoffrey Rose's work has been valuable for the development of preventive epidemiology (5).

Today, epidemiology plays a vital role in determining biomarkers of various diseases including cardiometabolic diseases. Cardiometabolic diseases have a substantial effect at both the individual level and society level, yet there is still a great need for better knowledge. In the present thesis, new potential biomarkers for prediction of cardiometabolic disease were evaluated in population-based studies. This thesis is also an effort to highlight new possibilities for understanding underlying biological and pathophysiological mechanisms.

Arterial stiffness

Historical context of arterial stiffness

In extreme old age, the arteries themselves, the grand instrument of the circulation, by the continual apposition of earth, become hard, and as it were bony, till, having lost the power of contracting themselves they can no longer propel the blood, even through the largest channels, in consequence of which death naturally ensues.

–John Wesley, 1703–1791

The interest in vascular health can be dated back to ancient times. The relation between hardness of the pulse and disease has been described as early as 200 B.C. in the ancient Chinese text of "the yellow emperor's classic of internal medicine"(6). Later on in the 17th century, Thomas Sydenham recognised the impact ageing had on vascular health by expressing that "a man is as old as his arteries"(7).

One of the earliest works to understand the functioning of the arteries was carried out by physicists like Young (1808), who discovered the pressure wave speed for incompressible liquids contained in elastic tubes. Furthermore, hydraulic and elastic theory on arteries were put forward by Poiseuille (1840), Moens (1878), and Korteweg (1878)(8). Later on, the analysis of the pulse wave was developed in the nineteenth century by Mahomed (1872). He improved the sphygmograph, a device used to measure blood pressure, originally made by Karl von Vierordt (9).

There has been immense development with regards to measurements of arterial stiffness as well as great interest in the role of vascular ageing in health over the last decade (10). However, despite increasingly sophisticated measurement techniques, modern therapies remain limited regarding treatment of increased arterial stiffness.

Histological properties of arteries

A thorough understanding of the hemodynamics and mechanics of the circulatory system is essential to gain insight into the various measures of arterial stiffness. The arterial wall histologically comprises of three distinct layers. These are highly specialized to perform their individual functions. The innermost layer is the tunica intima, which is primarily composed of a single layer of simple squamous endothelial cells, surrounded by a connective tissue basement membrane with elastic fibres. The endothelial cells synthesize and secrete a wide variety of substances including those that regulate vascular tone, enzymes that control blood clotting, immune responses and inflammation (11, 12). The middle layer, the tunica media, is made up of vascular smooth muscle cells (VSMCs), elastic tissue consisting of elastin and collagen. The medial layer is usually the thickest layer but its thickness varies across the different vascular beds. It not only provides support for the vessel but also plays a role in the regulation of blood flow and blood pressure by changing the vessel lumen sizes (13). The outermost layer of the vessel wall is chiefly composed of collagen, and is called the tunica externa or tunica adventitia (14). The three layers of the arterial vessel wall are illustrated in **Figure 1**.

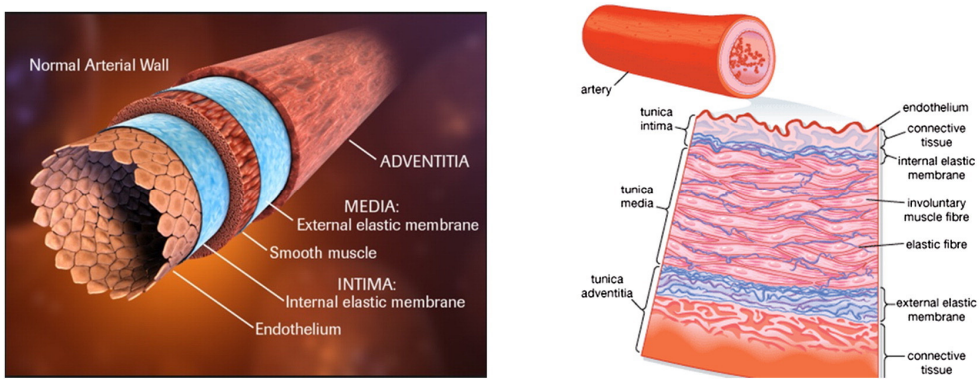

Figure 1:
Schematic representation of distinct layers of arterial wall. Left: top view of layers in artery, and right: cross-sectional view of layers in artery. Reproduced from Fortier et al. IJC Heart & Vessels 2014(15). Copyright © 2014, with permission from Elsevier.

The tunic media is the most important layer in large arteries, and the main determinant of arterial stiffness. In large arteries, the components of the medial layer are organized in concentric elastin sheets, interweaved by VSMCs, extra cellular matrix, and collagen fibres. These provide mechanical strength to the artery. With progressively increasing distance from the heart, the composition of the arteries changes from being primarily

elastic and rich in elastin, to becoming more muscular and rich in VSMCs. This alteration in composition plays an essential role in the perfusion of organs.

Pathophysiology of the large arteries

The circulatory system consists centrally of the heart and proximally of large elastic arteries, which are different from the distal muscular arteries. Large central arteries, primarily the aorta, exert a strong cushioning function, which leads to a steady flow in the microvasculature. Thus, the aorta has the essential function of acting as a conduit for circulating blood from the left ventricle to the peripheral organs. Additionally, the large arteries also perform the important function of dampening the high-energy pulsations generated by the heartbeats and supporting the perfusion of organs. This volume-buffering effect is known as the *Windkessel* effect. The Windkessel model was developed by the quantitative physiologist Otto Frank in 1899 (16, 17) to explain the hemodynamics of the arterial system. The buffering function of the large arteries ensures a smooth and continuous blood flow to the peripheral circulation, thereby reducing cardiac work. The muscular or distributing arteries, in turn draw blood from elastic arteries and branch into a network of resistance vessels consisting of small arteries and arterioles. Whereas the large elastic arteries have an important role in dissipation of energy generated by the heart's contraction, the muscular arteries, having layers of smooth muscles, offer regulation of blood flow and perfusion.

In the circulatory system, a pressure wave is generated by ejection of the left ventricle, which propagates along the arterial tree. This is reflected in the peripheral vessels and returns as a backward running wave towards the heart. The reflected wave normally returns to the central aorta in late systole and early diastole, and augments perfusion pressure in the coronary circulation. Wave reflection limits transmission of pulsatility into the periphery, thereby preventing damage to the microcirculation (18).

With increasing age, the walls of large elastic arteries undergo adverse structural and functional changes, leading to arterial stiffness, also known as arteriosclerosis. This process is characterized by loss of elasticity and thickening of the walls of arteries. Loss of elastin is central to the process of arterial stiffness (19). In addition to ageing, various pathological states also profoundly affect the stiffness of large arteries, including diabetes, hypertension and chronic kidney disease (13, 20, 21). These disease states are associated with an accelerated process of arterial stiffness.

As the arteries become stiffer, this leads to a loss of their volume buffering function. The pulse wave travels quicker through the vessels and the wave reflection arrives

prematurely in systole rather than diastole. This leads to increase in left ventricular (LV) afterload (22, 23). As a result, there is LV hypertrophy, increased oxygen demand and eventually LV failure. This consequently leads to cardiovascular disease (CVD) development (24). Arterial stiffness also ultimately leads to end organ damage as a result of disturbance of flow to microcirculation.

A summary of the differences between a compliant and stiff aorta as seen in health and in disease state is shown in **Figure 2.**

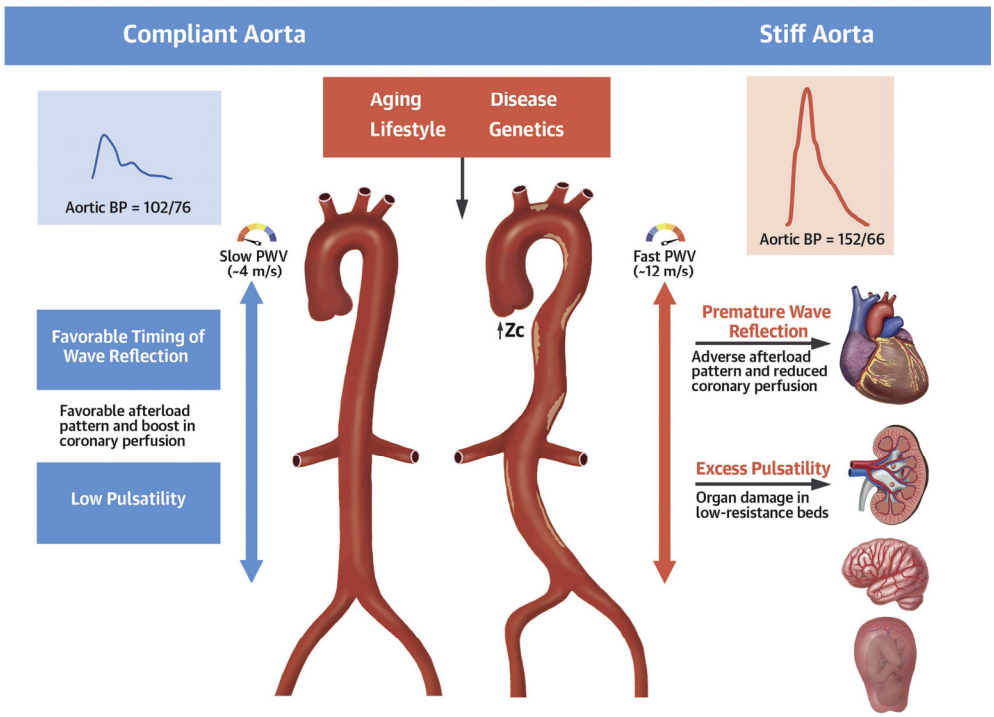

Figure 2:
Role of large artery siffness in health and in disease. Reproduced from Chirinos et al. Large-Artery Stiffness in Health and Disease: JACC State-of-the-Art Review. Journal of the American College of Cardiology. 2019;74(9):1237-63(25). Copyright © 2019, with permission from Elsevier Science & Technology Journals

Measures of arterial stiffness

Arterial stiffness has shown to be an important and independent predictor for adverse cardiovascular events and all-cause mortality (26, 27). Various indices have been

developed to measure arterial stiffness, which represent different characteristics of the vascular system (28).

Pulse wave velocity:

One of the important indices used to measure arterial stiffness is pulse wave velocity (PWV). The Bramwell-Hill equation explains the relationship between PWV and distensibility (29), by describing the theoretical link between the distensibility of the tube and the propagation speed of the wave in a log and uniform tube.

PWV is calculated as the ratio of the distance between two arterial recording sites and the time delay between them. The wave forms are measured non-invasively and transcutaneously by using different techniques such as distension, pressure or Doppler (8). The time, Δt, can be measured between the feet of the two waveforms. This is known as the foot-to-foot method, as illustrated in **Figure 3**. The distance between the two measuring sites is ΔL. PWV is then measured as a ratio using the equation PWV=ΔL /Δt.

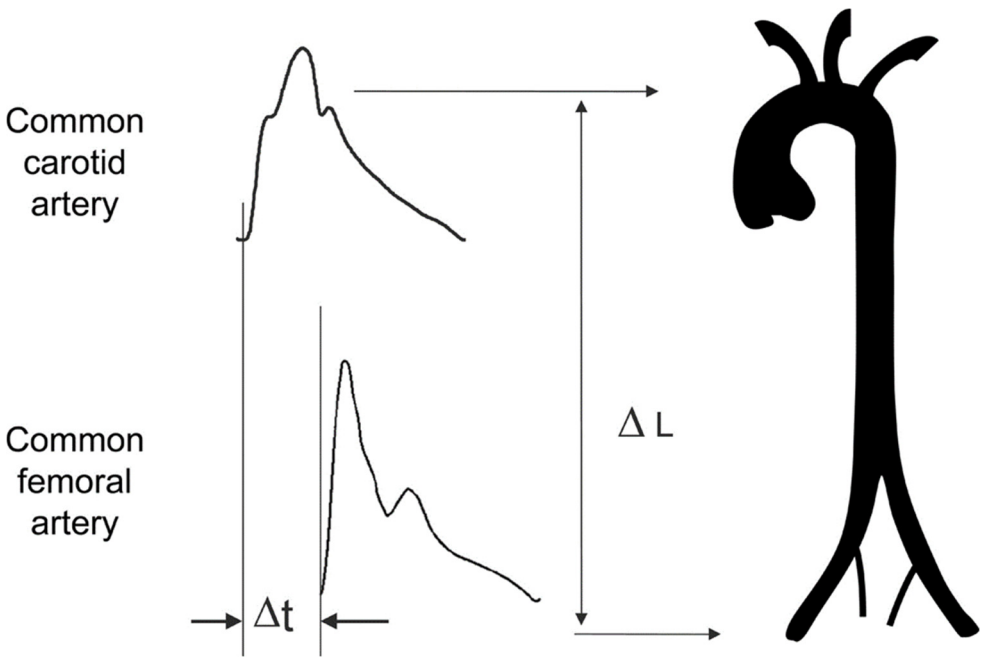

Figure 3:
Foot to foot method of measurement of c-f PWV. Laurent S, et al. Eur Heart J. 2006 (8). Reproduced with permission from Oxford University Press.

Among the various indices developed, the most widely accepted and considered as the gold standard is the carotid femoral pulse wave velocity (c-f PWV) (8). Here the pulse

wave is measured between two points, the carotid and femoral artery (typically on the right side), and covers the carotid–aortic–iliac–femoral pathway. Therefore, c-f PWV is considered to be a direct measurement of the regional arterial stiffness.

Pulse wave velocity can also be measured between other arterial sites. One example is the brachial-ankle pulse wave velocity (b-a PWV), which, as the name indicates, is measured between the brachial and the tibial artery. However, this measurement also includes the peripheral muscular arteries, which differ from the central arteries in the age related process of stiffening. Hence, c-f PWV and b-a PWV are not interchangeable. On the other hand, indices such as central pulse pressure, augmentation index are indirect, surrogate measures of arterial stiffness, and provide additional information related to wave reflections (8). In the Framingham heart study, when evaluated along with other indices for arterial stiffness assessment, c-f PWV proved to be a better predictor for cardiovascular risk (30).

The unit of PWV is typically presented as metre per second (m/s). Reference values for PWV for the European population were published in 2010 (31). One of the points to keep in mind while calculating c-f PWV is to specify the distance measured between the carotid and femoral arteries, as this can differ depending on the device used. A c-f PWV value of more than 10 m/s is considered high. However, this cut off applies when the distance is calculated as direct distance between the two measuring points, multiplied by 0.8. Distance measured by this method is known as the direct method, as it is considered to estimate the true arterial distance (32).

Risk factors for arterial stiffness

Age is considered one of the most important determinants of arterial stiffness (33). The central arteries undergo many changes during the life span of an individual. The age-related increase in arterial stiffness has been shown to be more pronounced in chiefly the major arteries rather than the peripheral vessels (18).

Blood pressure is also an important risk factor for the development of arterial stiffness. Hypertension leads to increased luminal pressure which triggers the transition towards more collagen than elastin in the arteries, ultimately resulting in stiffer arteries (34). Central obesity and dyslipidaemia have shown to be strong determinants of arterial stiffness (35, 36). Systemic inflammation has been shown to be associated with increased arterial stiffness (37, 38). Studies have shown that there is increased arterial stiffness in subjects with chronic inflammatory conditions (39-42), indicating that they are closely related. Metabolic changes such as hyperglycaemia also impact stiffness of

arteries, resulting in an accelerated process above normal vascular ageing (43). This association could be explained by increased glycation of arterial wall proteins in hyperglycaemia, leading to stiffer arteries (44).

Difference between atherosclerosis and arteriosclerosis

Both arteriosclerosis and atherosclerosis are important pathological aspects of the blood vessel effecting it in different ways. These two terms are often used interchangeably, and often the process of arterial stiffening is paralleled by atherosclerosis. However, it should be noted that arteriosclerosis and atherosclerosis are pathologically and mechanistically distinct processes. Whilst arteriosclerosis is focused on morphological changes in the tunica media, atherosclerosis is characterized by deposition of lipids, foam cells and calcium in the tunica intima (45).

Arteriosclerosis is one of the earliest manifestations of adverse structural and functional changes in the large arteries. Subclinical CVD can be assessed by markers of atherosclerosis, including coronary artery calcification score (CACS) and intima-media thickness (IMT) (46). CACS is used to evaluate subclinical atherosclerosis as a surrogate endpoint. Recent studies have suggested that CACS is of predictive value, particularly in asymptomatic subjects (47-49). Both arterial stiffness and CACS are important predictors for adverse cardiovascular outcomes in the general population. It would be worthwhile to explore how these two parameters relate to each other.

Diabetes

Historical overview of diabetes

Man may be the captain of his fate, but he is also the victim of his blood sugar.

-Wilfrid G. Oakley, 1905 - 1998

Diabetes was first described in 1550 B.C. in an Egyptian medical text, the Ebers Papyrus, as a condition in which a person passed too much urine and lost weight (50). Later on, in the first century, Aretaeus coined the term "diabetes" meaning "a siphon" in Greek to describe the disease as "melting down of flesh and limbs into urine". During the 5th and 6th century A.D., Indian physicians such as Sushruta described two forms of diabetes, one observed in older, obese people, and the other in thin people who did not survive for long (51-53). In the 17th century, the English physician Thomas Willis (1621-1675) remarked on the sweetness of the urine (54), and added the word 'Mellitus' to the name, which is the Latin word for honey, leading to urine tasting as a means of diagnosis (50). Today diabetes is broadly classified into type 1, type 2, and gestational diabetes. However, further classification of type 2 diabetes has now been developed (55).

Over time, there has been tremendous effort to understand the mechanisms leading to diabetes, yet it is still one of the major global health concerns.

Description of diabetes

Diabetes mellitus is a multifactorial disease, usually characterized by either elevated fasting or postprandial blood glucose levels, or both. Depending on the aetiology, hyperglycaemia occurs as a consequence of decreased insulin secretion from pancreatic β-cells, increase in insulin resistance, or a combination of both. Moreover, studies show that type 2 diabetes develops as a result of an interaction between genetic and

environmental factors, which consequently leads to insulin resistance or pancreatic β-cell dysfunction (56, 57).

According to the definition of the World Health Organization (WHO), diagnosis of type 2 diabetes is made if there is a fasting venous plasma glucose level ≥7.0 mmol/L or plasma glucose 2 hours after a standard oral glucose tolerance test (OGTT) is ≥ 11.1 mmol/L. In certain circumstances, an elevated HbA1c can also be used for the diagnosis of diabetes (58). The diagnostic criteria is presented in **Figure 4**.

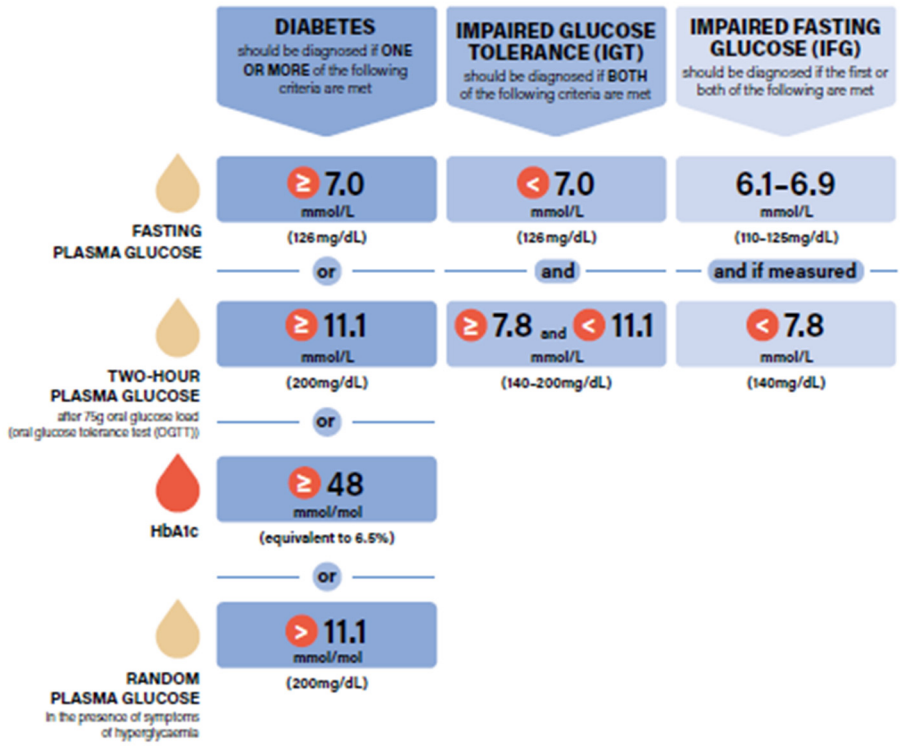

Figure 4:
Diagnostic criteria for diabetes. Reproduced from International Diabetes Federation. IDF Diabetes Atlas, 9th edn. Brussels, Belgium; 2019 (59). Copyright © 2019 with permission from IDF

Epidemiology of diabetes

Diabetes is an ever-growing global health problem of utmost priority. The most prevalent type of diabetes is type 2 diabetes, accounting for more than 85% of the total diabetes prevalence (60). Globally, the impact of diabetes is understood by monitoring its prevalence. According to the International Diabetes Federation (IDF), in 2019, among people aged 20-79 years, 463 million people (9.3%) were living with diabetes. It is predicted that by 2045, this number will rise to an overwhelming 700 million (10.9%)(59). The prevalence of diabetes globally, as well as in different regions of the world, is shown in **Figure 5**. In Sweden, the estimated age-adjusted prevalence of diabetes was reported to be 4.8 % in 2019 (among individuals aged 20-79 years) (59).

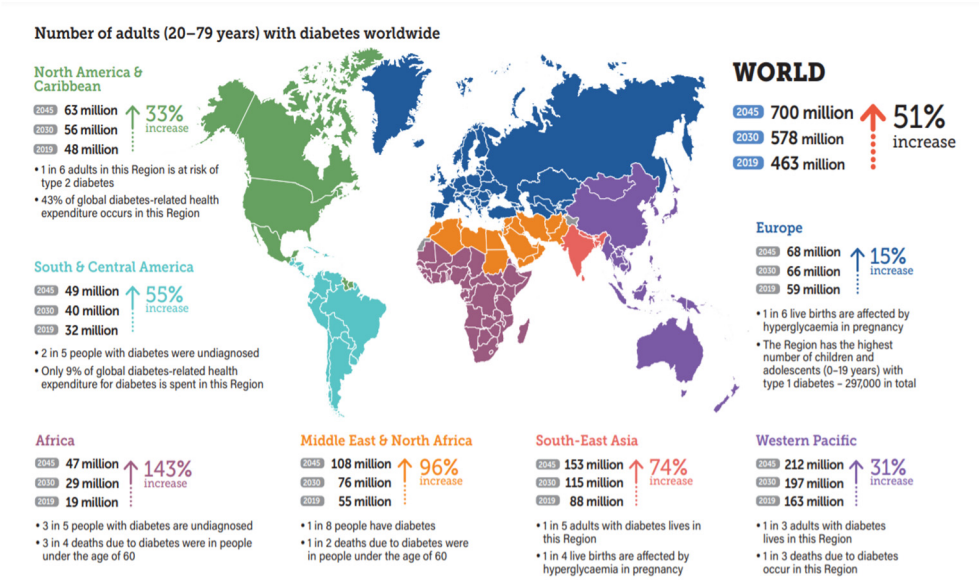

Figure 5:
Global prevalence of diabetes. Reproduced from International Diabetes Federation. IDF Diabetes Atlas, 9th edn. Brussels, Belgium; 2019 (59). Copyright © 2019 with permission from IDF

The steady increase in diabetes prevalence can be attributed to population growth, increase in an ageing global population, urbanization, increasing prevalence of obesity and physical inactivity.

Pathophysiology of diabetes

Type 2 diabetes results from impaired insulin release (β cell dysfunction) and/or reduced insulin sensitivity, which alters glucose uptake in the peripheral organs (insulin resistance). The body still produces insulin but it is insufficient to meet the needs. While the exact mechanism is still unclear, genetics, environmental factors, and risk factors (obesity, smoking, increased low-density lipoprotein (LDL)) are considered to be associated with an increased risk of diabetes development.

Risk factors for diabetes

Given the immense burden of disease imposed by diabetes, a plethora of research has explored the effect of risk factors associated with diabetes. These risk factors vary depending upon the aetiology of diabetes, and can be classified as either modifiable or non-modifiable risk factors.

Non-modifiable risk factors for type 2 diabetes include ethnicity, genetics, and age. Type 2 diabetes is polygenic, meaning more than one gene is responsible for the disease. Ageing is associated with an increased risk for the development of type 2 diabetes as a consequence of the combined effects of increased insulin resistance and impaired pancreatic islets function (61). Family history is another important risk factor. A positive family history of diabetes has shown to result in a 2.4 fold increased risk for type 2 diabetes (62, 63).

Modifiable risk factors of diabetes include hypertension, smoking, physical inactivity, unhealthy dietary habits, hyperlipidaemia, and obesity. Among the modifiable risk factors, obesity is one of the strongest and well-established risk factors (64). When assessing obesity, body mass index (BMI) is commonly used, but studies have indicated that waist circumference is a better predictor of type 2 diabetes (65). Physical inactivity and unhealthy diets are increasing worldwide, and are both directly and indirectly (through increased prevalence of obesity) affecting the global prevalence of diabetes. Hypertension has also been shown to be associated with an increased risk of diabetes (66, 67). Smoking is another modifiable risk factor that has been associated with higher risk of type 2 diabetes (68, 69). The suggested underlying mechanism is that smoking leads to increased insulin resistance and thereby an increased risk of diabetes.

Biomarkers

A biological marker or biomarker is defined as "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention"(70). Essentially, biomarkers can serve as an important tool to give insight for diagnosis, early identification, prognosis or pharmacological response to therapy of a disease. Biomarkers can be further classified into circulating, genetic and tissue biomarkers (71). Biomarkers can include simple markers such as age, blood pressure, BMI, circulating parameters (such as cholesterol, lipids), imaging biomarkers and more complex genetic biomarkers (72). A summary of the role of biomarkers is presented in **Table 1**.

Table 1: Summary of the role of biomarkers

Surrogate end points	A substitute for clinical outcomes
Diagnostic tools	For early detection of disease
Prognostic indicators	Provides information on the course of the disease
Response monitoring	To monitor the response of the patient to targeted therapy
Predictors	To identify subpopulations of patients that are likely at higher risk of disease
Risk assessment	To identify factors to evaluate susceptibility to disease
Screening/detection	To indicate the presence of disease

Biomarkers do not essentially have a causal association with an outcome of interest i.e. association does not mean causality. However, they can provide us insight related to the pathogenesis and risk of developing a disease. Therefore, a biomarker can be a *risk marker* associated with the prediction of a disease or, a *risk factor* linked causally to a disease (73).

Biomarkers of cardiometabolic risk

Biomarkers have been in use in clinical practice for decades, and play an essential role in preventive and diagnostic medicine. Blood-based or urinary biomarkers can be utilized in measuring the future risk of cardiometabolic disease, and in understanding the possible aetiological pathways. At present, there are many published risk scores for diabetes and CVD, but they constitute of mostly traditional risk factors, that is to say, age, gender, BMI, blood pressure, family history of disease and ethnicity. Generally, all such scores perform suitably for risk prediction (78). However, identification of novel biomarkers is essential for understanding the unknown mechanistic links and for identifying potential therapeutic intervention sites. They also improve the risk profile of those at increased risk. Moreover, important causal pathways can be uncovered by understanding the links.

Low-grade inflammation or chronic inflammation

Inflammation is a self-protective reaction of the body, and normal biological response against harmful stimuli such as injury and infections. Under normal conditions, inflammation ends after the clearance of the injurious agents and infection. The inflammatory pathways are regulated during this process in order to limit tissue damage. However, if it persists, inflammation can lead to tissue damage. This state is known as chronic or low-grade inflammation. Chronic inflammation has been closely linked with numerous non-communicable diseases including diabetes, CVD, cancer, rheumatoid arthritis and chronic obstructive lung disease (74). The importance of inflammatory dysregulation in chronic diseases is recognised, yet the exact mechanisms underlying these disorders are not fully elucidated. Chronic inflammation can be characterized by various proteins, which can be monitored to reflect the inflammatory status.

Acute-phase proteins and diabetes

Acute-phase proteins either increase or decrease in plasma concentration in response to inflammation. Chronic inflammation has been shown to be associated with the development of insulin resistance and diabetes (75-77). The relationship between development of diabetes and various inflammatory proteins has been explored in studies previously, though the results have been inconsistent (78-82). As inflammation is considered an important pathway leading to diabetes, therefore, exploring important biomarkers in this regard would be helpful.

Acute-phase proteins and arterial stiffness

Inflammation is known to play a contributing role in atherothrombosis. It is also accepted to play an essential role in stiffening of large arteries. The process of chronic inflammation is also related to other parallel processes such as atherosclerosis, endothelial dysfunction and smooth muscle cell migration (83). Many studies have explored the association between arterial stiffness and inflammation in subjects with chronic inflammatory conditions such as rheumatoid arthritis, systemic lupus erythematosus, inflammatory bowel disease and systemic sclerosis (39-42). Exploring the association in the general population can shed more knowledge.

Proteomics and diabetes

Research has been done to identify risk factors for diabetes (84), but the mechanism remains somewhat unclear. Inflammatory factors appear to have a role in the development of diabetes. Therefore, it is plausible to think that changes in protein structure, function or levels could be related to the underlying mechanism. Understanding of pathological mechanisms leading to the development of diabetes at the molecular level is important for the prevention and therapeutic intervention (85, 86). Proteomics is the large-scale study of proteins, which aims to quantify and characterize proteins playing a role in the biological processes of the disease. The use of Multiplex proteomic platform to find candidate biomarkers for risk prediction for various diseases has increased in recent years (87). Generally, a panel of candidate biomarkers can be selected to detect association of the protein biomarkers with diseases. Since epidemiological studies have reported that diabetes is associated with an enhanced risk of different types of cancers (88), oncology related proteins might be an interesting candidate. Association between proteins related to diabetes and cancer is not widely explored. Some, not all, of the associated increased risk between these two states could be attributed to the shared risk factors. Research is needed to elucidate the linking mechanism.

Biomarker of vascular health and diabetes

It is known that diabetes is characterized by increase in the threshold of blood glucose concentration, which predisposes individuals to microvascular and macrovascular complications. This can be due to the accelerated process of arterial stiffness in diabetes (43). The elevated risk of CVD is nearly two-fold in individuals with diabetes (89), which may be contributed to increased stiffness. It is already known that arterial stiffness is a well-established predictor for CVD, and is associated with end organ

damage (90). However, evidence has suggested the concept that arterial stiffness can be a possible risk marker for diabetes itself. The relationship between risk of development of diabetes and various hemodynamic parameters of large artery stiffness, such as pulse pressure and central aortic pressure, has been assessed in some studies (91, 92). Taken together, these studies indicate that perturbed vascular health may affect the pancreas, which eventually can be reflected in the form of glucose metabolism disturbance, and enhance the risk of diabetes. Therefore, use of c-f PWV, a direct measure of arterial stiffness, as a tissue biomarker in relation to diabetes could prove to be useful in earlier identification of high-risk individuals and allow new avenues to explore appropriate intervention.

Main Hypothesis and theoretical framework development

Cardiometabolic diseases are one of the leading non-communicable diseases in the world and share many risk factors. These diseases are different entities, but they are not completely exclusive and show many links (93). The overall aim of the present thesis is to explore known and novel biomarkers in relation to cardiometabolic disease. Low-grade inflammation is known to confer an increased risk for both diabetes and CVD. This has been shown by previous studies. However, there are discrepancies in results, and certain pathways are either unclear or not widely explored. One of the aims of the present thesis was, thus, to examine the association of circulating inflammatory biomarkers with the risk of incident diabetes and arterial stiffness. This would help to understand their additional risk prediction ability beyond that from traditional clinical risk factors.

We also aimed to explore the lesser known but plausible links between arterial stiffness, a tissue biomarker, and diabetes, as well as CACS, a marker of sub-clinical atherosclerosis. We intended to highlight knowledge gaps in the existing literature related to the predictive and aetiological value of these biomarkers. The ultimate aim is to improve the knowledge of novel biomarkers to understand pathophysiological pathways.

In summary, we intended to study the link between various biomarkers and cardiometabolic disease to identify various potential pathways, including inflammatory pathways and proteins pathways. The main theoretical framework of the thesis is illustrated in **Figure 6.**

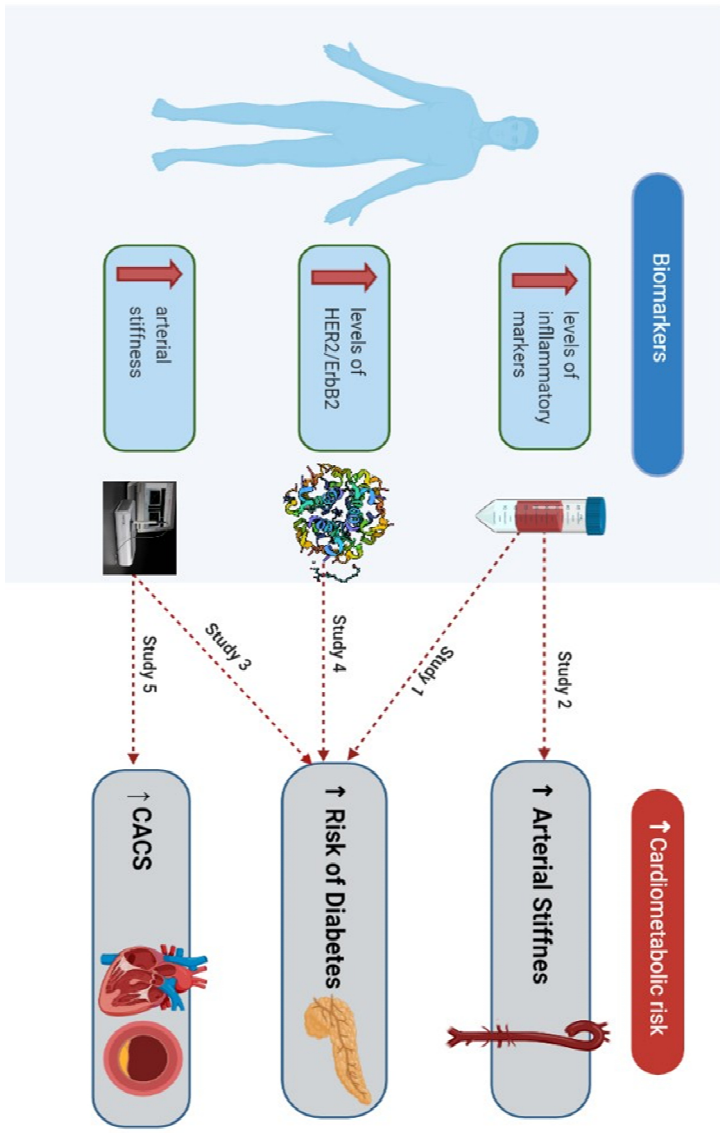

Figure 6:
Graphical presentation of the hypothesized framework of the studies included in the thesis

Aims

The overall aim of this project is to investigate the association between biomarkers and risk of development of diabetes, increased arterial stiffness and atherosclerosis. The specific aims of this doctoral thesis are as follows:

I. To study the association between acute-phase proteins and incidence of diabetes

II. To study the association between acute-phase proteins and arterial stiffness

III. To study the association between arterial stiffness and incidence of diabetes

IV. To explore the relationship between HER2/ErbB2 and incidence of diabetes

V. To assess the association between arterial stiffness and CACS, a measure of sub-clinical atherosclerosis

Methods

Study populations

The Malmö Diet and Cancer study-Cardiovascular cohort

The Malmö diet and cancer study (MDCS) is a large prospective population based study comprising of men and women from the city of Malmö, southern Sweden. It is part of the European Prospective Investigation into Cancer (EPIC) organized by IARC, Lyon, France (94). The primary objective of the MDCS was to study the relation between diet, lifestyle factors and risk of cancer (95, 96). All men, born 1923-1945, and all women, born 1923-1950, living in Malmö were invited to participate in the MDCS. Two means of recruitment were used in parallel: passive recruitment directed towards the community as a whole using advertisements in public places and local newspapers, and active recruitment by personal invitation letters (97). Recruitment was carried out between January 1991 and September 1996. There were 74,138 individuals, of which 68,905 were eligible for the study, and 30,446 responded and were initially included. A total of 28,449 (11,246 men and 17,203 women) had complete baseline examinations and 28,098 had also complete diet assessment. This corresponds to a participation of 40.8% (for men 38.3% and for women 42.6%) (96). The exclusion criteria for participation in the MDCS were mental retardation or inadequate Swedish language skills.

The baseline investigations of the MDCS comprised of a self-administered extensive questionnaire, anthropometric measurements, and collection of blood samples. The questionnaire included questions regarding socioeconomic factors, social support, occupation, disease history, medication usage and lifestyle factors such as physical activity, smoking habits and alcohol intake. Non-fasting blood samples were drawn, which were separated and stored in the biological bank at -80 °C (98, 99).

Between November 1991 and February 1994, a random 50 % sample of study subjects from the MDCS (every other participant) was invited to take part in a study with the objective to investigate the epidemiology of carotid artery (100). 6103 individuals

accepted, of whom 5540 were re-scheduled for fasting blood samples. This sub-cohort is referred as the MDCS-cardiovascular cohort (MDCS-CC).

Re-examination of the MDCS-CC

Between May 2007 and September 2012, the MDCS-CC underwent a re-examination, in which 3734 individuals [women= 2212 (59.2 %), men =1522 (40.8%)] participated. This was 76% attendance of the eligible population (101). The reasons for non-participation were either sickness, emigration or unwillingness (n=1333) or death before scheduled visit (n= 1036). Risk factors at the re-examination were evaluated by using laboratory tests, physical examination, and a questionnaire. Fasting blood samples were stored at -80°C. Arterial stiffness measurements were carried out for the first time in the MDCS-CC re-examination.

The MDCS, MDCS-CC and the MDCS-CC re-examination were approved by the Lund University Ethical Committee (Baseline ID LU-5190, re-examination ID 532-2006). The studies complied with the Declaration of Helsinki, and all the participants provided written informed consent. The MDCS-CC and the re-examination were used for analyses in **papers I-IV**, and the study flow charts of the participants for these studies are presented in **Figure 7 and 8.**

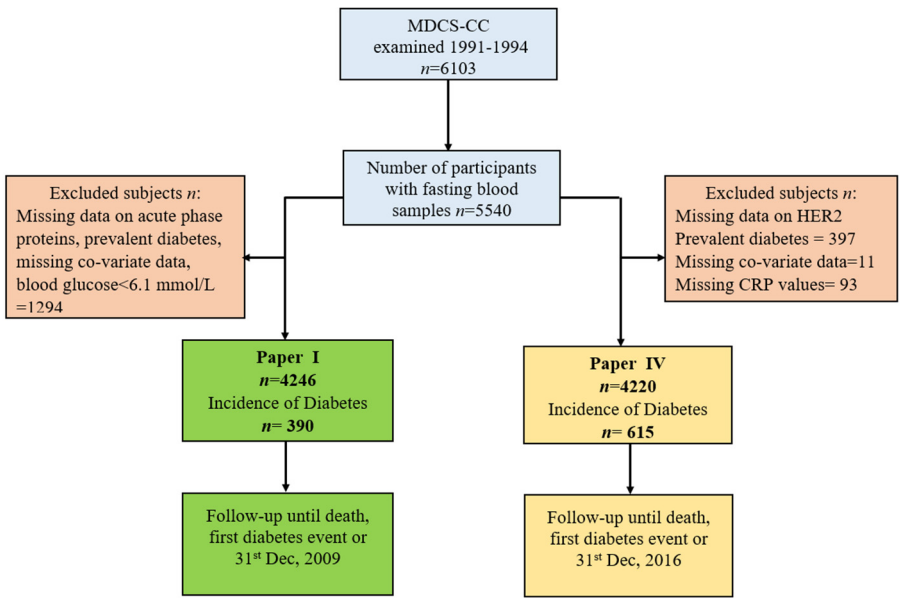

Figure 7:
Study flow chart for papers I and IV

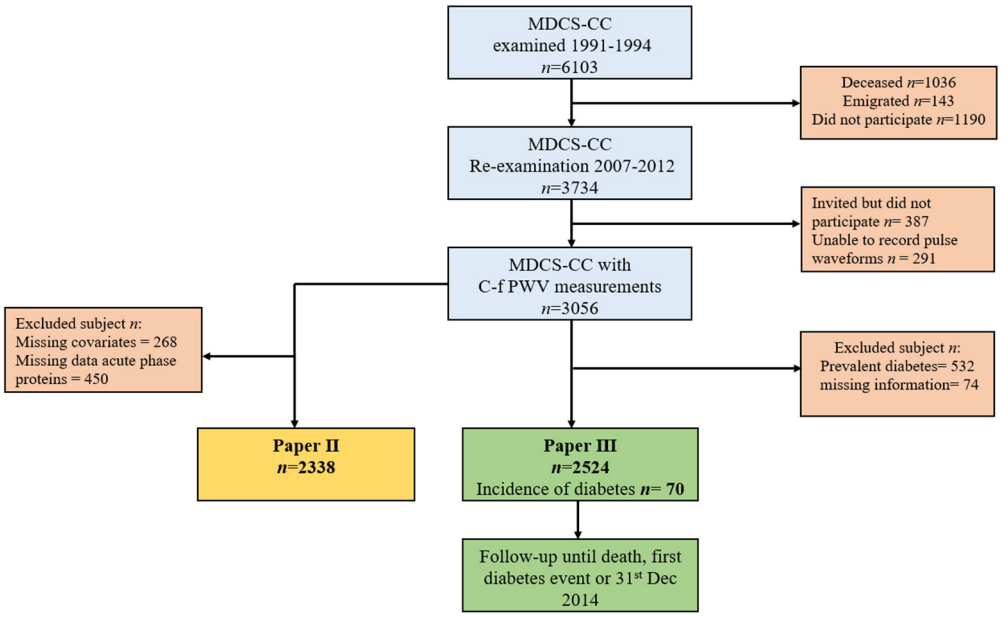

Figure 8:
Study flow chart for papers Ⅱ and Ⅲ

The Swedish Cardiopulmonary Bioimage Study (SCAPIS) cohort

The Swedish Cardiopulmonary Bioimage Study (SCAPIS) is a collaborative multicentre project conducted at six Swedish university hospitals (Gothenburg, Linköping, Malmö/Lund, Stockholm, Umeå and Uppsala) (102). The study was designed with the aim to predict and prevent CVD and chronic obstructive pulmonary disease (COPD), and provides a nationwide, open-access, population-based cohort for research.

A pilot trial of SCAPIS was carried out in Gothenburg, Sweden, from February 2012 to November 2012, to check the feasibility of the study. The participants of the pilot study were not included in the SCAPIS cohort.

By random selection from the Swedish population register, 30,154 participants aged 50-64 years, including both men and women, were enrolled. The overall participation rate for SCAPIS was 49.5%. The study included completion of a comprehensive questionnaire, biochemistry analysis, anthropometric measurements and extensive imaging. Some measurements were unique to a centre and were not carried out at other

sites. The c-f PWV measurements were conducted in Malmö (6251 individuals, 53% participation rate) and Linköping (5057 individuals, 58% participation rate).

The study was approved by the Regional Ethical Review Board in Umeå (2010-228-31M), Lund (2016/1031) and Linköping (2018/478-31). Written informed consent was obtained from the participants. The study flow chart for the study population from the SCAPIS cohort for **paper V** is illustrated in **Figure 9.**

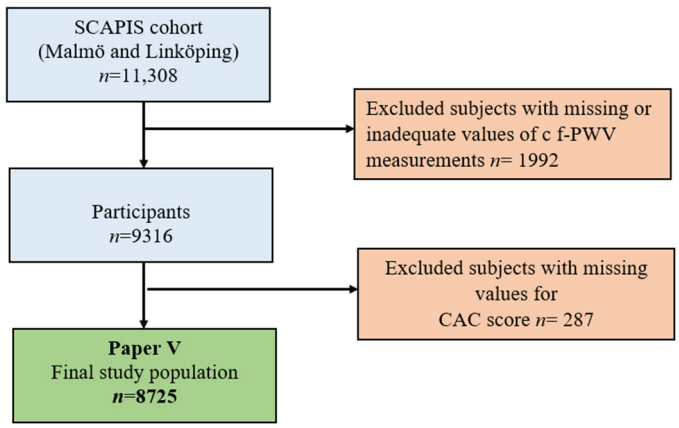

Figure 9:
Study flow chart for paper V

An over view of the studies included in this thesis is presented in **Table 2**.

Table 2: Summary of the studies included in this thesis

Paper	Paper I	Paper II	Paper III	Paper IV	Paper V
Study population	MDCS-CC	MDCS-CC and Re-examination	MDCS-CC Re-examination	MDCS-CC	SCAPIS
Study Design	Prospective cohort	Prospective cohort	Prospective cohort	Prospective cohort	Cross-sectional cohort
Main exposure	Acute-phase proteins	Acute-phase proteins	c-f PWV	HER2/ErbB2	c-f PWV
Main outcome	Diabetes Incidence	Arterial stiffness	Diabetes Incidence	Diabetes Incidence	CACS
Mean Follow-up (years)	15.6 ± 3.4	16.9 ± 1.5	4.43 ± 1.40	20.20 ± 5.90	
Statistics	Cox regression, Kaplan-Meier curve	Linear regression	Cox regression, Kaplan-Meier curve	Cox regression, Kaplan-Meier curve	Multinomial logistic regression

Assessment of exposures

Paper I and Paper II

In **paper I** and **II**, acute-phase proteins from the MDCS-CC were the exposure of interest. These were measured in the venous blood samples, which were taken in the morning after fasting overnight and frozen at –80°C immediately after collection. High-sensitive CRP in plasma was analysed using the Tina-quant® CRP latex assay (Roche Diagnostics, Basel, Switzerland). Plasma levels of ceruloplasmin, orosomucoid, haptoglobin, alpha-1-antitrypsin and C3 were analyzed using Cobas c-systems (Roche Diagnostics GmbH, Germany). The reference values were 0.15–0.30 g/l for men and 0.16–0.45 g/l for women for ceruloplasmin. For alpha-1-antitrypsin, the reference value was 0.9–2.0 g/l. The reference value for orosomucoid was 0.5–1.2 g/l. Finally, the reference value for haptoglobin was 0.3–2.0 g/l (www.roche.com).

Paper III

The measurements for the c-f PWV were carried out in MDCS-CC re-examination on average 261 days after the first visit in the follow-up examination. These were performed non-invasively using applanation tonometry (SphygmoCor, Atcor Medical, Australia) following a specific study protocol. The participants were asked to refrain from coffee (3 hours), alcohol (12 hours) and smoking (4 hours) before the examination. The measurements were done with the participants in supine position after five minutes of resting. Blood pressure measurements were also performed just before measuring the c-f PWV using the OMRON M5-1 IntelliSense device. The carotid artery and femoral artery measuring points were marked. Pulse curves from the carotid and femoral arteries were obtained with a pressure-sensitive probe. The distance was measured from the suprasternal notch to the umbilicus, and from the umbilicus to the measuring point at the femoral artery minus the distance from suprasternal notch to the measuring point at the carotid artery. The distance was measured with a customized tape with two slider indicators. The distance measured here by subtraction method is an indirect method for measurement. The direct method is done by multiplying the total measured carotid-femoral distance by 0.8, as recommended by a current expert consensus document (32). PWV was monitored over 10 s and mean PWV was calculated for the pulses during this time. Each participant had varying number of successful measurements, ranging between one and five. The aim was to obtain three measurements each, which was possible in 86.7% of the cases. Mean c-f PWV used in the analysis was calculated from these measurements. Mean arterial

pressure (MAP) was determined using the formula (2 × diastolic pressure + systolic pressure)/3. The mean coefficient of variation between the c-f PWV measurements in the same individual was 6.3% (±SD 4.4%).

The inter-observer variability between two technicians was evaluated twice in 17 and 13 participants. Inter-observer difference was 5.0% (± SD4.0) and 7.2% (±SD9.9), respectively.

Paper IV

Circulating HER2/ ErbB2 was analysed in the plasma from the blood samples acquired during the baseline examinations (1991–1994) and stored at -80°C until the analysis in 2015. The protein was measured with the Olink Proseek Multiplex Oncology I $V2^{96\times96}$ assay at the Clinical Biomarkers Facility, Science for Life Laboratory, Uppsala, Sweden. HER2/ErbB2 levels were expressed as normalized protein expression values as arbitrary units on a log2 scale. The lower limit of quantification was 0.95 pg/mL and the upper limit was 15,625 pg/mL. The intra-assay (within a run) and the interassay (between runs) coefficient of variation value were 5% and 21%, respectively.

Paper V

For **paper V**, data from SCAPIS cohort was used. In the SCAPIS cohort, similar protocol for arterial stiffness measurement was followed. C-f PWV was measured using the device Sphygmocor Xcel (Atcor Medical, Australia)(103). Participants were advised to refrain from caffeine, heavy meal for 3 hours, nicotine for 4 hours and alcohol for 12 hours prior to the examination. The examination began after five minutes of rest in supine position. Blood pressure cuffs were attached to the upper left arm and to the right thigh approximately 10-20 centimetres below the groin. The distance was measured from the carotid pulse to the upper edge of the thigh cuff, and from the femoral pulse to upper edge of the thigh cuff. Both these distances were multiplied by 0.8. The final distance was calculated by subtracting the distance between the femoral pulse and the upper margin of the thigh cuff from the distance between the carotid receptor and the thigh cuff (32). This distance was divided by the time difference between the signal registered from the tonometer at the carotid artery and simultaneous capturing of the signal from the femoral cuff, to obtain c-f PWV measurement.

At each assessment, c-f PWV was measured twice. If the difference between the two measurements was >0.5 m/s, a third measurement was done. The participants with two values for c-f PWV were included in the study and the average mean of the measurements was used in the analysis. MAP was calculated as (2 × diastolic pressure +

systolic pressure)/3, where the systolic and diastolic pressures were measured before the c-f PWV measurement.

To test reliability, test-retest approach was used. After one year, 154 participants underwent repeated measurements of c-f PWV. Out of these, 152 participants had c-f PWV values available from the original examination. The mean values (± SD) for the original c-f PWV and repeat c-f PWV were 8.14 (± 1.14) and 8.70 (± 1.33), respectively and showed acceptable reliability (Pearson Correlation: 0.75, p <0.001). The inter-class correlation coefficient (ICC) was used to determine the repeated measures reliability. The estimated average measures ICC provided evidence of good test–retest reliability for original c-f PWV and repeat c-f PWV (ICC = 0.806, 95%CI: 0.567-0.895, p < 0.001).

Assessment of covariates

Data from MDCS-CC was used for papers I, II and IV.

Information on age, family history of diabetes, current use of anti-hypertensive or antidiabetic medications, smoking habits, leisure-time physical activity and educational level was obtained from the questionnaire. Trained nurses carried out anthropometric measurements, with the subjects wearing light indoor clothing and no shoes. Standing height (centimetres) was measured with a fixed stadiometer. Weight (kilograms) was measured by using a calibrated balance beam scale. BMI was calculated as weight (kilograms) divided by the square of height (meters). Waist circumference (centimetres) was measured midway between the lowest rib margin and the iliac crest. Participants were classified into four categories regarding smoking habits: current smokers, occasional smokers, ex-smokers, and non-smokers. Low level of leisure-time physical activity was defined as the lowest quartile of a score revealed through 18 questions covering a range of activities in the four seasons. Educational level was divided into three categories: school years <9, 9–12 and<12, respectively.

Fasting cholesterol and glucose levels were analysed on fresh blood samples according to standard procedures at the Department of Clinical Chemistry, Skåne University Hospital, Malmö, Sweden. HbA1c was measured by ion exchange chromatography. The reference values in nondiabetic individuals were 3.9–5.3 % (19–34 mmol/mol).

Data from MDCS-CC re-examination was used for papers II and III.

Again, information was gathered-using questionnaire related to smoking habits, use of antidiabetic medications, antihypertensive treatment, and family history of diabetes. Blood pressure was measured after 10 min of rest in supine position using the OMRON M5-1 IntelliSense device. Blood samples were collected after an overnight fast (20). Fasting plasma glucose (FPG) was determined using HemoCue (HemoCue AB, Ängelholm, Sweden). The examination also consisted of an OGTT after an overnight fast, with measurement of plasma glucose before and 120 min after intake of 75 g glucose.

Data from SCAPIS cohort was used for paper V.

Waist circumference (cm) was measured in a similar manner, midway between the lowest rib margin and the iliac crest. Venous blood was drawn after over-night fasting and was immediately analyzed for LDL, high-density lipoprotein (HDL) and CRP. Brachial systolic blood pressure readings were taken before the c-f PWV measurements using an automatic device (Omron M10-IT) in the left arm after 5 min of rest in supine position. Information regarding smoking status, use of anti-hypertensive and anti-lipid medication was obtained from the questionnaire. Prevalent diabetes was determined by report of a prior diagnosis of diabetes in the questionnaire or new cases identified at baseline.

Assessment of outcome

Paper I, III and IV

Incident diabetes was the primary outcome of **papers I, III and IV**. New onset diabetes was assessed during the follow-up period starting from the date of enrolment until the end of follow-up, emigration, death or incidence of diabetes. Incident diabetes cases were identified by linkages to various local and national registers. Briefly, diabetes was ascertained from six sources: the Swedish National Diabetes Register, the regional Diabetes 2000 register of the Scania region, the Malmö HbA1c register, the Swedish inpatient register, the Swedish outpatient register, and the nationwide Swedish drug prescription register. In the Swedish National Diabetes Register and the Diabetes 2000 register, new cases of diabetes were diagnosed according to established criteria (fasting plasma glucose concentration ≥7.0 mmol/L resulting from two repeated tests on separate occasions). In the Malmö HbA1c register, subjects were considered to have developed diabetes if they had at least two HbA1c recordings ≥42 mmol/mol (6.0%),

based on the Swedish Mono-S-based standardization system (corresponding to 53 mmol/mol [7.0%], according to the U.S. National Glycohemoglobin Standardization Program). In the Swedish inpatient and outpatient registers, diabetes was identified by diagnosis of a senior physician. A filled prescription of insulin or antidiabetic medication (Anatomical Therapeutic Chemical Classification code A10) was required for diagnosis in the nationwide prescription register.

All individuals with diabetes at the baseline examination were excluded from the papers related to analysis of incidence of diabetes. Additionally, for **paper III**, all individuals with a diagnosis of diabetes according to national or local registers prior to the c-f PWV examination in 2007–2012 were classified as prevalent, and were excluded.

Paper II

Arterial stiffness was the primary outcome in **paper II**. The same measurements of regional arterial stiffness with c-f PWV previously described as exposure for **paper III** above were used in **paper II**.

Paper V

The main outcome of **paper V** was coronary artery calcium score (CACS), a measure of sub-clinical atherosclerosis. Computed tomography (CT) was used to determine CACS. Non-contrast CT was performed using a dedicated dual-source CT scanner equipped with a Stellar Detector (Somatom Definition Flash, Siemens Medical Solution, Forchheim, Germany) (104). Participants were excluded from CACS measurement in case of presence of cardiac stent or history of by-pass surgery. Calcium content in each artery was measured and summed utilizing the scoring system elaborated by Agatston *et al* (105-107).

Study design and Statistical analysis

Statistical analyses were performed using IBM SPSS (Windows) version 22.0 for **papers I-IV** and version 24.0 for **paper V**, and STATA version 12 (StataCorp, College Station, Texas, USA). Two sided p-values of < 0.05 were regarded as statistically significant.

Baseline measurements

Standard descriptive statistics were used to summarize baseline characteristics for the cohorts. The baseline characteristics of the exposure group were compared using one way analysis of variance (ANOVA) for continuous variables and Pearson's chi-square test for categorical variables in the papers. Additionally for **paper II**, to test the differences between these groups, analysis of covariance (ANCOVA) and logistic regression were used for continuous and categorical variables, respectively and adjusted for age, sex, heart rate and MAP at follow-up.

In all analyses, skewed variables were natural logarithm transformed to achieve normal distribution.

Cohort studies

In epidemiology, a cohort study is a form of observational study design where no intervention by investigator is carried out. Broadly, a cohort is defined as "any designated group of individuals who are followed or traced over a period of time"(108). Cohort studies can be prospective or retrospective in design. In both designs, the exposed and unexposed populations are compared. In the prospective cohort study, an investigation is carried out before the outcomes of interest have been developed. Information about exposures is collected as they occur during the study. The groups are then followed "longitudinally" over a period of time into the future and incidence of outcome of interest is measured. On the other hand, in a retrospective design, the study is carried out in the present. Some people have already developed the outcome of interest and information about exposure is obtained from past records.

Analytical methods are used to explore the association between outcome and exposure. Measures of association with confidence intervals (CI) indicate the strength, direction, and range of an effect along with the likelihood of chance occurrence. For **paper I-IV**, a prospective study design was used.

Cross-sectional studies

Cross-sectional study design is where both exposure and outcomes are measured at the same time. It can be described as the snapshot of the population status in relation to the exposures and outcomes. Cross-sectional studies are conducted either before planning a cohort study or as a baseline for a cohort study. This type of study design can measure prevalence of disease or allows calculation of the odds ratio (OR) as a measure of association, but does not give information about incidence. Time sequence

of events is difficult to ascertain in cross-sectional studies, and, therefore, they cannot determine cause and effect. However, they can offer insights into correlations present at that particular point in time. Cross-sectional studies are relatively quick as compared with a cohort study. They provide descriptive analyses and provide grounds for hypothesis generation for investigating many exposure and outcome variables. However, the time sequence of events is difficult to ascertain in such studies. For **paper V**, cross-sectional data from SCAPIS was used.

Survival analysis

Survival analysis refers to a set of statistical techniques used to investigate the time it takes for an event of interest to occur. The specifically used methods for analysis of time-to-event data include the Kaplan-Meier estimate (non- parametric), log-rank test, and Cox regression (semi-parametric).

Cox proportional hazard regression

James Cox developed the Cox regression in 1972 for survival analysis (109). Cox regression builds a predictive model for time-to-event data. It provides a survival function for the prediction of the probability that the event of interest has occurred at a given time, ***t***, for a given set of the predictor variables. In survival analysis, each individual contributes time-at-risk from inclusion until experiencing the event of interest. All individuals do not suffer the event of interest, but they contribute essential information. This information is used for the estimate of the probability of event. An important aspect of survival analysis is censoring. Right censoring occurs when a subject exits the study before the event of interest occurs, or the study ends before the event has occurred.

As Cox regression is a time-to-event analysis, the time-scale has to be defined. The time scale used for calculating time-to-event can be time on study, the attained age of the subjects, or calendar time (110). In all our Cox regression analysis, time to follow-up has been used as the time scale. The regression coefficients obtained from Cox regression are the hazard ratios (HRs) for the exposure variables of interest. It is a type of risk ratio that estimates the relative survival (or failure) in one group when compared to another (the reference group).

Proportional hazard assumption

When modelling a Cox model, a key assumption to keep in mind is the *proportional hazards assumption*. The Cox model assumes that the effect of an exposure is constant over time, known as the proportional hazards assumption. When using Cox regression

for analysis, this assumption should be tested (111). Different methods can be used, which include graphical methods to visually check the assumption as well as statistical techniques. Graphical methods include plotting the log-transformed values of the Cox survival function over time (-log-log plots), and then visually inspecting if the curves for exposed and unexposed are parallel. If they are not parallel, a violation of the assumption is considered. Another way to test the proportional hazards assumption is use of Schoenfeld residuals test which is a goodness of fit test. This involves plotting of scaled Schoenfeld residuals with time and testing the correlation between these and time. If the Schoenfeld residuals are not correlated with time, the assumption is valid. The proportionality assumption can also be tested by using the time-dependent covariate terms in the analysis. The time-dependent covariate is a multiplicative term constructed as a function of time and the covariate or exposure of interest. This is introduced in the Cox regression model and the significance level for the time-dependent covariate coefficient in the Cox regression model is checked. If the coefficient of the product term (time and covariate being tested) is non-significant, it means that the proportional hazards assumptions have been met.

Kaplan-Meier curve

The most common non-parametric technique for modelling the survival function is the Kaplan-Meier estimate. The Kaplan-Meier curve is described as the probability to survive in a given length of time while considering time in many small intervals (112). Kaplan-Meier is commonly plotted for graphical presentation. The log-rank test can be used to statistically compare survival in different exposure groups.

The survival analysis approach was used for **papers I, III and IV**. Proportional hazard assumptions were tested for all cox regression analysis.

Linear regression

Linear regression is used to measure the association between a continuous dependent variable and one or more independent (predictor) variables, assuming a linear relationship. Simple linear regression model has one independent variable whereas a multiple linear regression model has more than one independent variables. The regression coefficient (β) estimates the change in the dependent variable for a one unit change in the predictor, keeping the effect of other variables constant in a multivariable linear regression. In **paper II,** linear regression was used to test for association between a continuous dependent variable (arterial stiffness) and other independent variables.

Multinomial logistic regression

Logistic regression is the appropriate analytical method to use when the dependent variable is categorical. Most commonly used logistic regression is binomial logistic regression, when the outcome is binary. Multinomial logistic regression is a type of logistic regression used for the prediction of a nominal dependent variable with more than two levels, and one or more independent variables. It is sometimes considered an extension of binomial logistic regression. One level of the dependent variable is selected as the reference category. The parameter estimates are relative to the reference group. The interpretation of the multinomial logit is that for each unit change in the independent variable, the logit of being in a particular category (of the outcome variable) relative to the reference group is expected to change by its respective parameter estimate, provided the other variables in the model are held constant. The OR and 95% CI are estimated based on the independent variables. In **paper V**, the main outcome was CACS which was divided into three categories and the lowest category was used as reference for analysis.

Selection of covariates

Covariates used in multiple regression analysis were selected based on previous literature and were decided beforehand. We attempted to control for covariates that were related to both the exposure and the outcome of interest in each of the studies. Additionally, multivariable analyses including c-f PWV were always adjusted for MAP as MAP has an effect on the intrinsic elastic properties of the arterial wall (113) and should be taken into consideration. Analyses with c-f PWV were also adjusted for heart rate as higher heart rate can lead to higher arterial pressure, and consequently increased arterial stiffness (114).

Effect modification

Effect modification is also termed as *interaction* or *heterogeneity of effect*. It means that the strength of the association between the primary exposure and the outcome differs according to the level of another variable. This variable is known as the effect modifier. Rather than an arbitrary statistical phenomenon, effect modification is a biological occurrence in which the exposure has a different impact in different circumstances. Effect modification can be tested by including a multiplicative term between the primary exposure and the potential effect modifier in the regression model. A key distinction between confounding and effect modification is that in epidemiology, the aim is to eliminate the confounding, and to explain effect modification. One way of

dealing with effect modification is to evaluate the effect association separately for every level of the effect modifier i.e. stratifying the analyses.

Receiver operating characteristic (ROC)

ROC curve, also known as area under the curve (AUC) analysis is an established technique for evaluating how well a marker is able to discriminate between individuals who experience disease onset and individuals who do not (115, 116). Generally, a higher AUC value is an indicator of better risk marker performance. The AUC also reflects the probability of an individual with disease having a higher risk marker value as compared to a healthy individual. ROC curve analysis was done in **paper V** to evaluate the added value of arterial stiffness to other traditional risk markers for the prediction of higher CACS.

Results

Paper I

The main findings of **paper I** are that there was significantly positive association between orosomucoid, haptoglobin, and CRP and the risk of incident diabetes. However, after further adjustments for baseline fasting glucose, the association remained significant for CRP, but not for other inflammatory proteins.

Complete data on acute-phase proteins and key covariates was available for 4246 subjects. The baseline characteristics of the study subjects stratified for sex are shown in **Table 3**.

There were 390 cases of incident diabetes (181 men and 209 women) during a mean follow-up period 15.6±3.4 years. The incidence of diabetes was 7.19 and 5.09 per 1000 person-years in men and women, respectively.

Table 3: Baseline characteristics of the MDCS-CC among men and women (N = 4246)

Baseline Characetersitics	Men	Women
N	1664	2582
Incidence of diabetes n (n per 1000 p-y	181 (7.19)	209 (5.09)
Glucose (mmol/l)	5.01 ± 0.4	4.80 ± 0.4
Age at baseline (years)	57.63 ± 6.0	57.39 ± .5.9
Ceruloplasmin (g/l)	0.46 ± 0.10	0.54 ± 0.12
Alpha-1-antitrypsin (g/l)	1.19 ± 0.26	1.21 ± 0.26
Orosomucoid (g/l)	0.71 ± 0.21	0.69 ± 0.20
Haptoglobin (g/l)	1.30 ± 0.58	1.31 ± 0.51
CRP (mg/l) (GM [5th–95th percentiles])	1.35 (0.30–8.30)	1.29 (0.30–7.21)
Waist circumference (cm)	92.15 ± 9.4	76.18 ± 9.5
Low physical activity (%)	20.9	22.4
Current smoking status (%, current and occasional smokers)	26.1	25.6
Use of anti-hypertensive medication (%)	15.0	13.3
Systolic BP (mmHg)	142.4 ± 18.5	139.2 ± 18.9
HDL (mmol/l)	1.23 ± 0.30	1.53 ± 0.35
Low educational level (%)	46.4	43.3

Data are mean ± SD unless otherwise indicated
p-y person year

Men had slightly higher glucose levels, greater waist circumference, higher blood pressure, and used more anti-hypertensive medications. Women had higher mean ceruloplasmin levels than men.

Ceruloplasmin, orosomucoid, haptoglobin, and CRP were significantly and positively correlated with fasting glucose, after adjustment for age and sex as shown in **Table 4**. However, after further adjustment for other covariates, the association remained statistically significant for orosomucoid and haptoglobin.

Table 4: Correlations between acute-phase proteins and glucose in non-diabetic individuals

	Glucose (age, sex)	Glucose (risk factors)[a]
Ceruloplasmin	0.035**	0.007
Alpha-1-antitrypsin	-0.004	-0.004
Orosomucoid	0.152***	0.075***
Haptoglobin	0.146***	0.095***
LnCRP	0.105***	-0.001

Values are standardized beta coefficients, from a linear regression model with the acute-phase proteins as the dependent variable
* p< 0.05; ** p<0.01; *** p<0.001
Risk factors: adjusted for age, sex, waist circumference, smoking, use of anti-hypertensive medication, systolic BP, HDL, low educational level, and low physical activity

In **Table 5**, results are shown by sex-specific quartiles of the acute-phase proteins in relation to incidence of diabetes. Subjects in the fourth quartile compared to the first quartile of orosomucoid, haptoglobin and CRP had significantly higher risk of incidence of diabetes after adjusting for covariates in Model 1. However, after further adjustment for fasting glucose in Model 2, the association was no longer significant for orosomucoid and haptoglobin. The association for CRP was attenuated, but still significant.

Table 5: Incidence of diabetes in relation to sex-specific quartiles of acute-phase proteins

	Q1 (Reference)	Q2	Q3	Q4	p-value trend
Orosomucoid					
Mean ± SD	0.46 ± 0.059	0.60 ± 0.00	0.74 ± 0.050	1.10 ± 0.163	
Incidence of diabetes n (n/1000)	53 (3.3)	79(5.5)	152(6.6)	106(8.4)	
Model 1	1	1.30 (0.91–1.87)	1.25 (0.90–1.74)	1.46 (1.03–2.08)	0.058
Model 2	1	1.16 (0.81–1.67)	1.06 (0.76–1.48)	1.18 (0.83–1.67)	0.510
Haptoglobin					
Mean ± SD	0.68 ± 0.182	1.07 ± 0.099	1.44 ± 0.110	2.05 ± 0.410	
Incidence of diabetes n (n/1000)	66 (4.1)	79 (4.5)	126(7.0)	119(8.2)	
Model 1	1	1.00 (0.71–1.40)	1.40 (1.03–1.92)	1.49 (1.08–2.05)	0.002
Model 2	1	0.91 (0.65–1.28)	1.23 (0.90–1.67)	1.19 (0.85–1.62)	0.100
CRP					
Mean ± SD	0.41 ± 0.149	0.92 ± 0.170	1.81 ± 0.380	6.44 ± 6.714	
Incidence of diabetes n (n/1000)	63(3.7)	72 (4.6)	104(6.4)	143(9.3)	
Model 1	1	1.10 (0.78–1.56)	1.17 (0.84–1.64)	1.44 (1.03–2.00)	0.023
Model 2	1	1.24 (0.87–1.76)	1.28 (0.92–1.79)	1.40 (1.01–1.95)	0.046

Model 1: Adjusted for age, sex, waist circumference, smoking, use of anti-hypertensive medication, systolic BP, HDL, low educational level, and low physical activity at baseline
Model 2: Adjusted for age, sex, waist circumference, smoking, use of anti-hypertensive medication, systolic BP, HDL, low educational level, low physical activity, and fasting glucose at baseline

The Kaplan-Meier survival curve for incident diabetes by quartiles of CRP is illustrated in **Figure 10,** showing a poorer diabetes free survival in participants with higher levels of CRP.

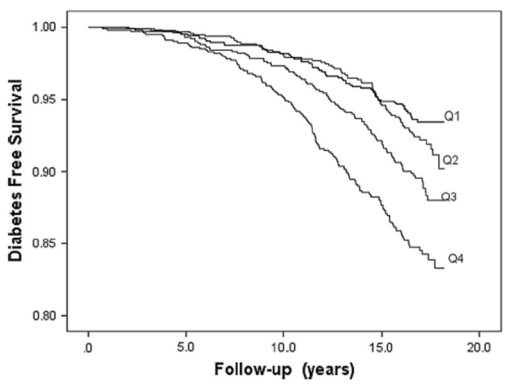

Figure 10:
Diabetes-free survival in relation to sex-specific quartiles (Q1–Q4) of CRP

Paper II

The mean follow-up period from the baseline measurements of the acute-phase proteins to the c-f PWV measurements was 16.9±1.5 years. **Table 6** displays the baseline characteristics of the whole population as well as the comparisons of characteristics between participants with high (c-f PWV ≥12m/s), intermediate (c-f PWV<12 and ≥ 8m/s), and low arterial stiffness (c-f PWV<8m/s). Those with high arterial stiffness exhibited higher cardiovascular risks, except for smoking, than those with low arterial stiffness. Plasma levels of all the acute-phase proteins were higher in those with high arterial stiffness.

Table 6: Follow-up and baseline characteristics of the whole study population (n=2338) and comparisons of characteristics of participants with high, intermediate and low arterial stiffness

	Whole study population (n=2338)	High arterial stiffness (n=515)	Intermediate arterial stiffness (n=1525)	Low arterial stiffness (n=298)	p value**
Follow-up					
c-f PWV *(m/s)	10.03[8.80-11.73]	13.53[12.67-14.83]	9.8[8.97-10.70]	7.4[6.97-7.77]	
Age (years)	72.08(±5.56)	75.25(±5.10)	71.68(±5.36)	68.70(±4.53)	
Heart rate (beats/min)	62.76(±9.91)	65.27(±10.04)	62.64(±9.96)	59.04(±8.01)	
Mean arterial pressure (mmHg)	95.56(±10.49)	100.02(±10.62)	95.44(±9.95)	88.50(±8.89)	
Baseline					
Age (years)	55.97(±5.65)	59.21(±5.27)	55.56(±5.44)	52.50(±4.53)	0.112
Smoking-current smokers [n (%)]	398(17)	63(12.2)	273(17.9)	62(20.8)	0.230
Systolic blood pressure (mmHg)	137.58(±17.64)	145.48(±17.67)	136.33(±16.89)	130.28(±16.55)	<0.001
Waist (cm)	81.25(±11.60)	84.99(±12.29)	80.55(±11.31)	78.41(±10.32)	<0.001
HDL cholesterol(mmol/L)	1.43(±0.37)	1.38(±0.38)	1.44(±0.36)	1.46(±0.35)	0.002
LDL cholesterol (mmol/L)	4.09(±0.96)	4.30(±0.99)	4.05(±0.94)	3.91(±0.95)	0.015
Diabetes [n (%)]	121(5.2)	60(11.7)	55(3.6)	6(2)	<0.001
Lipid lowering drug medication [n (%)]	43(1.8)	17(3.3)	20(1.3)	6(2)	0.234
Anti-hypertensive medication [n (%)]	272(11.6)	95(18.4)	158(10.4)	19(6.4)	<0.001
Ceruloplasmin* (g/L)	0.49[0.43-0.56]	0.50[0.43-0.57]	0.48[0.43-0.56]	0.48[0.42-0.55]	0.009
Alpha-1-antitrypsin(g/L)	1.18(±0.25)	1.18(0.25)	1.18(0.24)	1.16(0.27)	0.321
Orosomucoid(g/L)	0.68(±0.20)	0.71(0.20)	0.67(0.19)	0.64(0.19)	<0.001
Haptoglobin(g/L)	1.25(±0.51)	1.31(0.51)	1.23(0.50)	1.20(0.52)	0.025
Complement C3*(g/L)	1.45[1.28-1.64]	1.54[1.35-1.72]	1.43[1.28-1.62]	1.37[1.19-1.58]	<0.001
CRP* (mg/L)	1.10 [0.60-2.30]	1.50[0.70-2.80]	1.10[0.60-2.20]	0.80[0.50-1.60]	<0.001

Values are expressed as means (±SD) or percentages unless specified elsewise. *median [25th - 75th percentile]
High arterial stiffness: c-f PWV ≥12 m/s; intermediate arterial stiffness: ≥8 m/s and <12m/s; Low arterial stiffness: c-f PWV <8 m/s
** p –values for differences between groups with high, intermediate and low arterial stiffness after adjustment for age, sex heart rate and MAP

The association of arterial stiffness is relation to the quartiles of acute-phase proteins is presented in **Table 7.** After adjusting for potential confounders in Model 2, the c-f PWV remained significantly higher for participants in the 4th quartile vs 1st quartile of alpha-1-antitrypsin (geometric mean: 10.32 m/s vs 10.04 m/s) (p<0.05), C3 (10.35 m/s vs 10.06 m/s) (p<0.05) and CRP (10.37 m/s vs 9.96 m/s) (p<0.001).

Table 7: Relationships between the acute-phase proteins and quartiles of c-f PWV

Model 1					
	Geometric means (n)	Geometric means (n)	Geometric means (n)	Geometric means (n)	
	Q1	Q2	Q3	Q4	p for trend
Ceruloplasmin	10.13 (558)	10.04 (606)	10.22 (597)	10.40* (577)	0.004
Alpha-1-antitrypsin	10.05 (595)	10.12 (505)	10.26 (673)	10.34** (565)	0.004
Orosomucoid	10.05 (645)	10.00 (531)	10.21 (577)	10.52*** (585)	<0.001
Haptoglobin	10.04 (633)	10.20 (562)	10.21 (559)	10.35** (584)	0.006
Complement-C3	9.89 (577)	10.08 (595)	10.27*** (586)	10.55*** (580)	<0.001
CRP	9.84 (534)	10.04 (610)	10.36*** (607)	10.52*** (587)	<0.001
Model 2					
	Q1	Q2	Q3	Q4	p for trend
Ceruloplasmin	10.18	10.05	10.21	10.36	0.035
Alpha-1-antitrypsin	10.04	10.16	10.27*	10.32*	0.007
Orosomucoid	10.20	10.03	10.18	10.37	0.058
Haptoglobin	10.12	10.26	10.17	10.25	0.440
Complement-C3	10.06	10.14	10.24	10.35*	0.012
CRP	9.96	10.10	10.33**	10.37***	<0.001

*p<0.05 **p<0.01 ***p<0.001
Ln transformed values were used to calculate the p-values with Q1 as the reference group.
Model 1: Adjusted for age, sex, heart rate, MAP at follow-up.
Model 2: Adjusted for age, sex, heart rate, MAP at follow-up, and baseline age, smoking habits, systolic blood pressure, HDL, LDL, waist circumference, diabetes, use of anti-hypertensive medication and use of lipid lowering medication

We also compared c-f PWV in individuals with and without diabetes at the follow-up examination. There were 305 individuals with diabetes and 2033 without diabetes at the re-examination. Diabetes was associated with higher c-f PWV even after adjusting for other co-variates and taking into account acute-phase proteins.

Arterial stiffness is known to be increased in individuals with rheumatic disease and CVD. Therefore, as part of the sensitivity analysis, individuals with rheumatic disease and CVD at follow-up were excluded. We also took into consideration pharmacological factors and excluded participants that were taking anti-inflammatory medication such as steroids (n = 7, 0.3%) and aspirin (n = 82, 3.5 %) at baseline. The results still showed significant association between alpha-1-antitrypsin, C3, CRP and arterial stiffness.

Paper III

All prevalent cases of diabetes before the date of measurements of c-f PWV (n=532) were excluded. The final study population included 2450 participants. During a mean follow-up of 4.4±1.40 years, there were 68 (2.8%) cases of diabetes. The crude incidence of diabetes (per 1,000 person-years) was 3.5, 5.7, and 9.5, respectively, for subjects in the first, second, and third tertiles of c-f PWV. The baseline characteristics of case subjects with prevalent diabetes are presented in **Table 8**. The table also shows comparison of characteristics included in the study population with and without diabetes. In subjects with diabetes, significantly higher c-f PWV, MAP, fasting plasma glucose (FPG), and waist circumference was reported.

Table 8: Characteristics of individuals with no diabetes, incident diabetes and prevalent diabetes

	Included in the study population		Prevalent diabetes (excluded)
	Diabetes free	**Incident diabetes**	
Number (n)	2382	68	532
c-f PWV * (m/s)	9.90(8.63-11.46)	10.95(9.76-12.70)***	11.23(9.63-13.05)
Age (years)	71.94(±5.54)	70.96(±6.05)	72.66(±5.30)
Heart rate (beats/min)	62.58(±9.82)	62.11(±9.10)	64.32(±10.82)
MAP (mmHg)	95.57(±10.51)	99.46(±12.24)**	95.84(±10.76)
Waist (cm)	90.24(±11.62)	96.21(±11.85)***	97.65(±12.40)†
Smokers n (%)	234(9.8)	7(10.3)	45(8.5)†
Use of antihypertensive drugs n (%)	1151(48.3)	49(72.1)***	422(79.3)
Fasting plasma glucose (mmol/L)	5.71(±0.60)	6.39(±0.70)***	7.75(±2.07)†
2-hour post OGTT plasma glucose (mmol/L)	6.68(±1.87)†	9.09(±2.59)†***	10.46(±3.71)† (*n*=235)
LDL cholesterol (mmol/L)	3.43(±0.90)	3.29(±0.87)	2.84(±0.92)†
HDL cholesterol (mmol/L)	1.47(±0.44)	1.27(±0.39)***	1.28(±0.38)†
Triglycerides (mmol/L)	0.90[0.70-1.20]	1.20[0.90-1.60]***	1.10[0.80-1.5]†
FH+ Father n (%)	174(7.3)	9(13.2)	73(13.7)
FH+ Mother n (%)	223(9.4)	14(20.6)**	111(20.9)

Values expressed are means (±SD) unless specified otherwise. *Median (25–75%). **P <0.01; ***P < 0.001, statistically significant difference in group with incident diabetes from group without diabetes. †Some individuals have missing data regarding these covariates. FH+, family history–positive

In **Table 9**, results are shown by tertiles of c-f PWV and per one SD increment of c-f PWV. The risk for diabetes was significantly higher for those in the highest tertile of c-f PWV. Taking into account potential confounders in the fully adjusted Model, the HR (95% CI) was 3.24 (95% CI 1.51–6.97) for those in the third tertile as compared to the participants in the first tertile.

Table 9: Incidence of diabetes in relation to tertiles (T1–T3) of c-f PWV (n = 2,450)

	T1	T2	T3	P for trend	P /SD
Number of participants	808	831	811	-	-
Incidence of diabetes n(n per 1000 p-y)	13(3.54)	21(5.70)	34(9.47)	-	-
Model 1	1	1.73(0.85-3.54)	3.41**(1.63-7.14)	0.001	<0.001
Model 2	1	1.83(0.88-3.80)	3.24**(1.51-6.97)	0.002	0.001
Men					
Number of participants	263	295	347	-	-
Incidence of diabetes n(n per 1000 p-y)	4(3.39)	8 (6.18)	16 (10.73)	-	-
Model 1	1	1.78 (0.52-6.10)	3.16 (0.90-11.06)	0.059	0.027
Model 2	1	1.38 (0.39-4.80)	2.37 (0.65-8.62)	0.151	0.089
Women					
Number of participants	545	536	464	-	-
Incidence of diabetes n(n per 1000 p-y)	9 (3.62)	13 (5.44)	18 (9.01)	-	-
Model 1	1	1.74 (0.72-4.18)	3.61**(1.44-9.09)	0.006	<0.001
Model 2	1	2.05 (0.83-5.05)	3.44*(1.30-9.10)	0.012	0.004

Model 1 is adjusted for age, sex, MAP, and average heart rate measured at the carotid artery. Model 2 is adjusted for age, sex, MAP, average heart rate measured at the carotid artery,waist circumference, smoking habits, FPG, LDL cholesterol, and antihypertensive drugmedication. *P<0.05; **P<0.01.
P for trend, P value for trend across tertiles; P/SD, P value per one SD increment of c-f PWV.

In the sensitivity analysis, effects of other traditional risk factors on the association was assessed by additional adjustments for classical risk factors including HDL cholesterol, triglycerides, 2-h post- OGTT plasma glucose (in place of fasting glucose), and family history of diabetes (mother and father). The HR decreased, but remained significant, for participants in the third tertile versus the first tertile of c-f PWV (HR 2.18 [95% CI: 1.003–4.719]).

Figure 11 shows the Kaplan-Meier curves for incidence of diabetes by different tertiles of c-f PWV, showing a decreasing probability of remaining diabetes free as the c-f PWV increases.

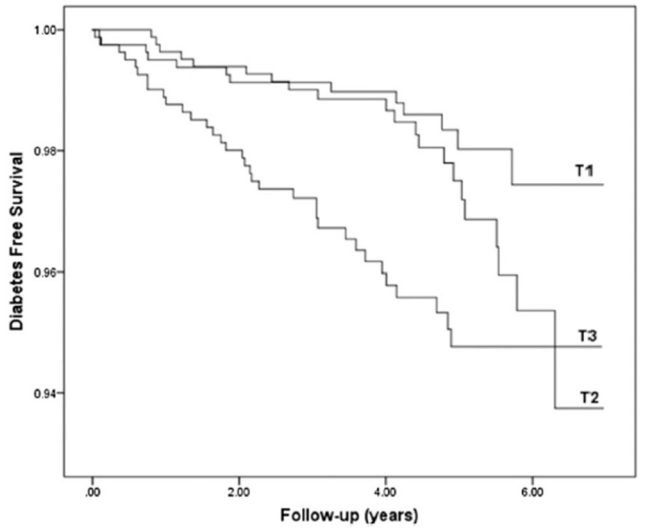

Figure 11:
Diabetes-free survival in relation to the tertiles (T1–T3) of c-f PWV

The study population was stratified into those with a normal fasting glucose (FPG <6.1 mmol/L) and impaired fasting glucose (IFG) (FP≥6.1 and≤6.9mmol/L) and those with normal glucose tolerance (OGTT <7.8 mmol/L) and impaired glucose tolerance (IGT) (OGTT ≥7.8 and ≤11.0 mmol/L). After adjusting for age, sex, MAP, and average heart rate, the risk of developing diabetes was significantly higher in both the prediabetes categories in participants with higher c-f PWV. The HRs for participants in the highest tertile of c-f PWV for the groups with IFG and IGT were 4.03 (95% CI 1.47–11.10) and 9.95 (95% CI 1.22–12.77), respectively.

Paper IV

The main findings of **paper IV** were that elevated levels of HER2/ErbB2 were associated with higher risk of diabetes. The study population consisted of 4,220 individuals. During a mean±SD follow-up period of 20.20±5.90 years, 615 participants (14.6%) were diagnosed with new-onset diabetes. The characteristics of the study participants across the quartiles of HER2/ErbB2 are presented in **Table 10**. Participants with elevated levels of HER2/ErbB2 generally had higher values of risk factors than those with lower levels.

Table 10: Relationships of risk factors across HER2/ErbB2 quartiles (n = 4,220)

	HER2/ErbB2				
	Q1	Q2	Q3	Q4	P value
Number (n)	1055	1055	1055	1055	
HER2/ErbB2 † (AU)	7.33(±0.26)	7.75(±0.07)	7.98(±0.07)	8.33(±0.18)	
CRP mg/L*	1.20(0.60-2.40)	1.10(0.60-2.30)	1.30(0.60-2.60)	1.50(0.70-3.10)	<0.001
Age (years)	56.42(±5.94)	57.48(±5.92)	57.60(±5.94)	57.79(±5.94)	<0.001
Sex (men) n (%)	265(25.1)	379(35.9)	441(41.8)	539(51.1)	<0.001
Waist circumference (cm)	78.60(±10.88)	81.40(±11.94)	83.14(±12.08)	86.44(±12.39)	<0.001
LDL (mmol/L)	3.99(±0.96)	4.11(±0.97)	4.20(±0.96)	4.36(±0.99)	<0.001
Fasting glucose (mmol/L)	4.75(±0.42)	4.87(±0.43)	4.89(±0.44)	5.01(±0.44)	<0.001
HbA_{1c}, %	4.68(±0.40)	4.78(±0.39)	4.81(±0.42)	4.85(±0.44)	<0.001
HbA_{1c}, (mmol/L)	28	29	29	29	<0.001
HOMA2IR* (n=4176)	0.70(0.50-0.90)	0.80(0.50-1.10)	0.80(0.50-1.2)	1.0(0.70-1.50)	<0.001
Insulin * (mIU/L) (n=4176)	5.0(4.0-7.0)	6.0(4.0-8.0)	6.0(4.0-9.0)	7.0(5.0-11.0)	<0.001
Systolic blood pressure (mmHg)	135.77 (±17.78)	139.29 (±18.41)	141.07 (±18.70)	144.50 (±18.54)	<0.001
BP lowering medication n (%)	128(12.1)	150(14.2)	150(14.2)	176(16.7)	0.030
Smoking habits n (%)					0.048
Never smokers	465(44.1)	447(42.4)	422(40)	396(37.5)	
Former smokers	385(36.5)	376(35.6)	404(38.3)	408(38.7)	
Current smokers	205(19.4)	232(22)	229(21.7)	251(23.8)	
Re-examination (2007-2012)					
Fasting glucose*(mmol/L) (n=2817)	5.7(5.3-6.1)	5.8(5.4-6.3)	5.8(5.4-6.3)	6.0(5.5-6.6)	<0.001
2-h post OGTT glucose * (mmol/L), (n=2637)	6.5(5.4-7.9)	6.6(5.4-8.0)	6.7(5.4-8.1)	7.3(6.0-9.0)	<0.001

Values are the mean ±SD, n (%), or *median (25th–75th percentiles) unless specified otherwise. AU, arbitrary units; BP, blood pressure. † Normalized protein expression values on a log2 scale.

The elevated levels of HER2/ErbB2 strongly correlated with measures of glucose metabolism. Baseline fasting glucose, HbA1c, HOMA2-IR, and insulin were all positively and significantly correlated with HER2/ErbB2 levels after adjusting for confounders. Moreover, we also explored the longitudinal association between HER2/ErbB2 levels, and fasting glucose and 2-h glucose at re-examination, which were significantly correlated, as shown in **Table 11**.

Table 11: Correlations between HER2/ErbB2 levels and measures of glucose metabolism at baseline and re-examination (2007–2012)

	Fasting glucose (baseline) (n= 4220)	HbA1c (baseline) (n=4220)	HOMA2-IR (baseline) (n=4176)	Insulin (baseline) (n=4176)	Fasting glucose (Re-exam) (n=2817)	OGTT (Re-exam) (n=2637)
Model 1	0.181***	0.157***	0.231***	0.225***	0.124 ***	0.109 ***
Model 2	0.130 ***	0.127***	0.202***	0.195***	0.083 ***	0.078 ***

Values are standardized beta coefficients from multiple linear regressions with HER2/ErbB2 as the dependent variable. Natural log-transformed values were used for HOMA2-IR, insulin, CRP, fasting glucose (at re-examination), and OGTT (at re-examination).
Model 1 was adjusted for age and sex.
Model 2 was adjusted for age, sex, waist circumference, smoking habits, systolic blood pressure, LDL cholesterol, use of antihypertensive medication, and CRP. ***P <0.001.

The adjusted risks for incident diabetes across the quartiles of HER2/ErbB2 are shown in **Table 12**. The participants in the 4th quartile had a significantly higher risk of developing diabetes than those in the lowest quartile. The risk remained significantly higher when adjusted for covariates in Model 2 (HR 1.72 [95% CI: 1.36–2.18]) and Model 3 (HR: 1.73 (95% CI: 1.37–2.19). The HR was attenuated, but still statistically significant when further adjusted for baseline fasting glucose (HR 1.31 [95% CI: 1.03–1.66]).

Table 12: Incidence of diabetes in relation to HER2/ErbB2 quartiles (n = 4,220)

HER2/ErbB2	Q1	Q2	Q3	Q4	P for trend	HR per 1 SD
Number of participants	1055	1055	1055	1055		
Incidence of diabetes n (per 1000 p-y)	111 (4.91)	127 (5.92)	145 (6.86)	232 (11.57)		
Hazards ratios						
Model 1	1	1.22 (0.94-1.57)	1.41 (1.10-1.81)**	2.41 (1.92-3.0)***	<0.001	1.52 (1.39-1.66)***
Model 2	1	1.08 (0.84-1.40)	1.19 (0.93-1.53)	1.72 (1.36-2.18)***	<0.001	1.25 (1.14-1.37)***
Model 3	1	1.09 (0.85-1.41)	1.19 (0.93-1.53)	1.73 (1.37-2.19)***	<0.001	1.26 (1.15-1.25)**
Model 4	1	0.93 (0.72-1.20)	0.99 (0.77-1.27)	1.31 (1.03-1.66)*	0.006	1.15 (1.05-1.25)**

Values are HR (95% CI) unless otherwise specified. Model 1 was the crude model. Model 2 was adjusted for age, sex, waist circumference, smoking habits, LDL cholesterol, systolic blood pressure, and antihypertensive drug medication. Model 3 was adjusted for model 2 variables and CRP. Model 4 was adjusted for model 3 variables and fasting glucose. HR per 1 SD; HR per 1 SD of the log2-transformed value. *P < 0.05; **P < 0.01; ***P < 0.001.

The Kaplan-Meier curves for incidence of diabetes across the quartiles of HER2/ErbB2 is presented in **Figure 12**.

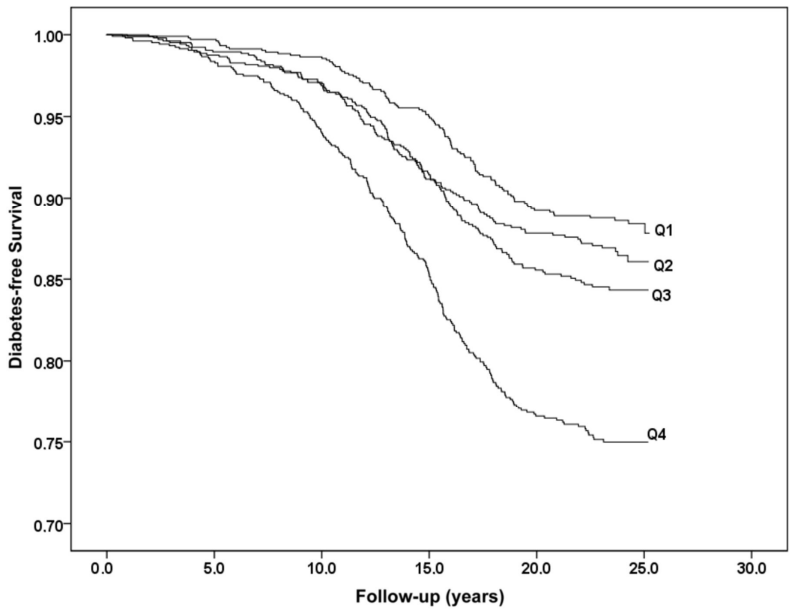

Figure 12:
Diabetes-free survival in relation to HER2/ ErbB2 quartiles

Paper V

In **paper V**, we explored the cross-sectional association between arterial stiffness and risk of development of CACS. The baseline characteristics of the study population across the four quartiles of c-f PWV are presented in **Table 13**. The mean value of c-f PWV was 8.36 m/s for the whole study population. Those in the highest quartile of c-f PWV had significantly higher mean age, MAP and systolic blood pressure, waist and heart rate, as compared to those in the lower quartiles. Moreover, there were higher proportions of participants with diabetes and those using anti-hypertensive and/or anti-lipid medication in the highest quartile. CACS was divided into three categories (≤ 10, > 10 and ≤ 100, and > 100) to present low, intermediate and high risk. Approximately 69.3%, 17.8% and 12.9% of the whole study population had CACS of ≤10, >10 and ≤100, and >100, respectively.

Table 13: Baseline characteristics

Characteristics	Whole population	Quartiles of c-f PWV				
		Q1	Q2	Q3	Q4	P value**
Number of participants (n)	8725	2170	2207	2177	2171	
Average cf-PWV (m/s)	8.36(7.6-9.25)	7.05(±0.46)	8.00(±0.21)	8.79(±0.25)	10.27(±0.97)	
Mean arterial pressure (mmHg)	93.67(86.67-101)	85.47(±8.31)	91.76(±8.22)	96.51(±8.85)	103.49(±9.94)	<0.001
Systolic blood pressure (mmHg)	128.10(±15.46)	116.17(±11.42)	124.62(±11.64)	130.70(±12.76)	140.94(±14.25)	<0.001
Average heart rate (beats /min)	61.07(±9.05)	58.54(±8.03)	60.13(±8.45)	61.76(±9.20)	63.88(±9.56)	<0.001
Age (years)	57.41(±4.339	55.94(±4.11)	56.99(±4.22)	57.71(±4.27)	59.03(±4.13)	<0.001
LDL-C (mmo/L)	3.51(±0.96)	3.49(±0.93)	3.50(±0.95	3.52(±0.96)	3.52(±0.99)	0.730
HDL-C (mmol/L)	1.67(±0.52)	1.80(±0.52)	1.70(±0.52)	1.63(±0.50)	1.55(±0.50)	<0.001
Waist (cm)	93.74(±12.65)	89.21(±11.96)	92.64(±12.21)	95.13(±12.43)	97.97(±12.32)	<0.001
CRP (mg/L)*	1.1(0.60-2.42)	0.90(0.60-1.90)	1.00(0.60-2.42)	1.10(0.60-2.42)	1.42(0.60-2.42)	<0.001
Men (n, %)	4138(47.4)	646(29.8)	946(42.9)	1122(51.5)	1424(65.6)	<0.001
Diabetes (n, %)	623(7.1)	69(3.2)	122(5.5)	162(7.4)	270(12.4)	<0.001
Anti-hypertensive drugs (n, %)	1648(18.9)	192(8.8	346(15.7	481(22.1)	629(29.0)	<0.001
Anti-lipid medication (n, %)	622(7.1)	88(4.1)	140(6.3)	175(8.0)	219(10.1)	<0.001
Smoking status (n, %)						0.242
current smokers	1275(14.6)	341(15.7)	323(14.6)	318(14.6)	293(13.5)	
CACS (n, %)						<0.001
≤ 10	6050(69.3)	1617(81.2)	1627(73.7)	1456(66.9)	1206(55.6)	
> 10 & ≤ 100	1553(17.8)	263(12.1)	359(16.3)	4296(19.7)	502(23.1)	
> 100	1122(12.9)	146(6.7)	221(10.0)	292(13.4)	436(21.3)	

Values expressed are means (±SD) or percentages unless specified otherwise.
*Median (25–75%); **P value is for difference across the quartiles of c-f PWV
LDL-C: low density lipoprotein cholesterol; HDL-C: high density lipoprotein cholesterol; CRP: C- reactive protein

The results of the analysis showed that as compared to having CACS ≤10 (reference category), the risk for the intermediate CACS category (>10 and ≤100) was greater for those in the 4th quartile of c-f PWV (OR: 1.66, 95% CI: 1.34-2.05) vs the 1st quartile (reference category) for Model 1 in the overall population. The results were quite similar and statistically significant for those in the 4th quartile when adjusted for further co-variates in Model 2 (OR: 1.58, 95% CI: 1.27-1.96) and Model 3 (OR: 1.57, 95% CI: 1.27-196). For high risk category of CACS >100, those in the 4th quartile of c-f PWV had significantly higher risk (OR: 1.78, 95% CI: 1.38-2.30) after adjusting for covariates in Model 1. The risk was attenuated but still statistically significant after further adjustments in Model 2 (OR: 1.64, 95% CI: 1.26-2.13). The results remained unchanged after adjusting for use of anti-hypertensive and anti-lipid medication in Model 3.

Similarly, 1 SD increase in c-f PWV was independently associated with a higher odds of having a CACS category >10 and ≤100 (OR: 1.15, 95% CI 1.06-1.25) and CACS category >100 (OR: 1.25, 95% CI 1.14-1.36) in the final multivariable model. The OR across the quartiles of c-f PWV for Model 3 for the intermediate and high CACS categories are presented in the **Figure 13**.

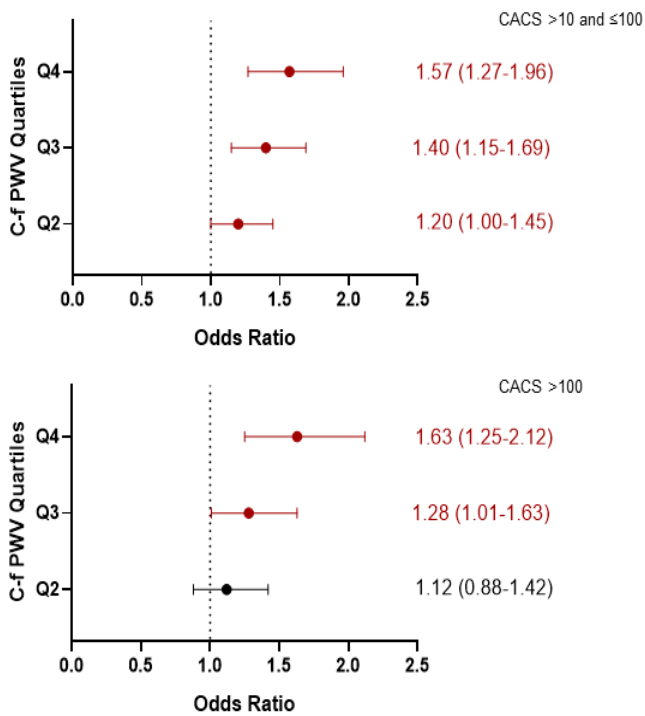

Figure 13:
Forest plot for OR across the quartiles of c-f PWV for CACS categories

Discussion

General Discussion

This thesis is a systematic effort to evaluate and understand biomarkers associated with increased cardiometabolic risk. The studies included in the present thesis were conducted in an epidemiological setting by using large population-based data sources that also enabled controlling for a wide array of recognised risk factors.

Acute-phase proteins and cardiometabolic risk predication

In **paper I** and **II**, we studied the association between chronic inflammation and cardiometabolic risk prediction. For this purpose, association between acute-phase proteins and incident diabetes, and risk of arterial stiffness was explored. The main findings of our study provide support to the existing knowledge that chronic inflammation is related to cardiometabolic risk in the general population.

Chronic Inflammation has been shown to be associated with metabolic disorders. Results of **paper I** showed that orosomucoid, haptoglobin and CRP were significantly associated with incidence of diabetes after adjusting for several confounders. However, when further adjustments were done for fasting glucose, the association was significant only for CRP. These findings are concordant with previously published results showing association between various inflammatory markers and incidence of diabetes (79-82) . A possible explanation for this link could be explained by obesity. Many studies have shown associations between obesity, acute-phase proteins and diabetes (117, 118). The presence of obesity and increased adipose tissue is considered a source of low-grade inflammation. In our study, this possible association was taken into account, and the analysis was adjusted for waist circumference. As the association was still significant, it can be speculated that there is a potential mechanism directly relating diabetes and inflammation. It should, however, be considered that previously it had been demonstrated that the association between CRP and diabetes is more likely to be non-causal, as shown in the study by Brunner et al (119).

The results of **paper II** showed that acute-phase proteins alpha-1-antitrypsin, C3 and CRP were associated with increased c-f PWV, even after controlling for traditional cardiovascular risk factors. This suggests that low-grade inflammation may play a role in the pathogenesis of arterial stiffening, which subsequently contributes to increased cardiovascular risk. The exact mechanism as to how inflammation modifies arterial stiffness is somewhat unclear. It should be noted that both chronic inflammation and arterial stiffness shared traditional risk factors, such as obesity and diabetes. Previous studies have looked at the link between inflammation and arterial stiffness, but these have consisted mostly of study populations with chronic inflammatory diseases. The results of our study indicate that systemic inflammation is a potential determinant for arterial stiffness in the general population as well.

Arterial stiffness and risk of diabetes

Paper III explored the temporal association between arterial stiffness and incidence of diabetes. The results of this study indicated that changes in vascular system can precede metabolic disturbances.

Very few previous population-based studies have looked at the association between different indices of measurement of large artery stiffness, and incidence of diabetes. Moreover, these studies have explored these parameters and their association with new-onset diabetes in high-risk hypertensive individuals only (92, 119). Association of c-f PWV with incidence of diabetes is a novel finding. One possible explanation for the association between arterial stiffness and incidence of diabetes could be offered by considering the cross-talk between arterial stiffness and endothelial dysfunction. It has been suggested that endothelial dysfunction can facilitate the development of diabetes (120). Endothelial dysfunction, in turn has been shown to be associated with increased arterial stiffness (121). Therefore, this could be a common pathway linking diabetes and arterial stiffness. Another plausible biological explanation is the perturbed microvascular function resulting from arterial stiffness. This may cause impaired tissue perfusion in the pancreas and ultimately contribute to the development of diabetes. Another point to consider is that raised glucose, even in the prediabetic range, contributes to stiffening of arteries. The analysis was, therefore, adjusted for plasma glucose levels (fasting and 2-h post-OGTT at the baseline examination), to take into account changes in the prediabetic range. Yet, this possibility cannot be completely excluded.

Proteomics and diabetes

In **paper IV**, a proteomic approach was used to identify proteins association with incident diabetes. An association between HER2/ErbB2 and incidence of diabetes was found, after adjusting for traditional confounders. To the best of our knowledge, this is the first study to explore the relationship between HER2/ErbB2 and incident diabetes.

HER2/ErbB2 is part of the epidermal growth factor receptor (EGFR) proteins, which are a family of receptor tyrosine kinases (122). This receptor family plays an essential role in normal development, and irregular expression or activation of the receptor family has been associated with many diseases, primarily progression of cancer (123).

HER2/ErbB2 is involved in regulating functions such as differentiation, proliferation, and apoptosis (124), and it plays a vital role in neural and cardiac development(125). The HER2 gene is amplified and the protein is overexpressed in 15–20% of breast cancers (126, 127). Its overexpression is correlated with poor prognosis and a more aggressive phenotype (128, 129). Levels of HER2/ErbB2 are monitored for the prognosis and the therapeutic management of breast cancers.

Epidemiological evidence links diabetes with an increased risk of certain types of cancer, such as those of the pancreas, liver, bladder, breast, endometrium, colon, and rectum (130). The association between these two conditions may partly be explained by some shared risk factors such as ageing, obesity, and diet. However, what remains unclear are the causes and biological mechanism of this link.

HER2/ErbB2 has mainly been studied in relation to its role in carcinogenesis. However, recent studies showing an association between HER2/ErbB2 and hyperglycaemia and insulin resistance suggest its role beyond oncogenesis (131, 132). Fernandez-Real et al. (132) explored and showed that serum HER2/ErbB2 levels were significantly associated with insulin resistance and decreased after weight loss in obese subjects, indicating that HER2/ErbB2 might have a role in the pathophysiology of diabetes (132). Similarly, a cross-sectional study by Memon et al. reported significant association between HER2/ErbB2 and hyperglycaemia and insulin resistance (131).

The potential mechanism behind this association is unclear. One plausible biological mechanism could be explained through fatty acid synthase (FASN) activity. Increased expression of FASN has been correlated with overexpression of HER2/ErbB2 (133). Some studies have explored the role of FASN as a possible surrogate marker for diabetes (134), and it has been associated with insulin resistance and type 2 diabetes (135). Therefore, it can be speculated that FASN mediates the effects of HER2/ErbB2 in the development of diabetes, or perhaps a synergistic effect occurs. Additionally,

HER2/ErbB2 has also been suggested to play a role in preadipocyte differentiation and therefore it can be speculated that the effect of HER2/ErbB2 on diabetes development is mediated though obesity (124). This close relationship with adiposity is intriguing with regard to the possible role of ErbB2 in diabetes development.

HER2/ErbB2 is a novel marker for diabetes. The findings are of importance from a mechanistic point of view, and provide suggestion for potential therapeutic interventions.

Arterial stiffness and CACS

In **paper V**, our main finding was that arterial stiffness, as determined by c-f PWV, is positively associated with increasing CACS.

To our knowledge, this is the largest epidemiological study to date exploring the relationship between arterial stiffness, using c-f PWV, and atherosclerosis. The results of our study are in line with several previous studies (136-141). However, these studies have explored the association between various other indices of arterial stiffness and CACS, or were conducted on a relatively smaller scale. The study of Kullo et al., explored the association between aortic PWV in a community based sample of around 400 participants, and demonstrated that CACS was positively associated with PWV (136). Similar results were observed in a study of around 800 participants in a cohort enriched for hypertension (137). The Rotterdam study explored the association between c-f PWV and CACS in elderly population (mean age = 71 years), and showed strong association of c-f PWV with coronary atherosclerosis (138).

Other studies have explored the association between b-a PWV and CACS, and have reported similar results (139, 140). In our study, use of a direct measure of arterial stiffness provides a clearer picture of the association between atherosclerosis and arteriosclerosis.

Methodological considerations

In an experimental study design, the investigators assign an exposure to a random sample of the study subjects to explore the effects of an exposure. On the other hand, in an observational study the investigators can only observe the effect of the exposure on the study subjects. This makes observational studies much more susceptible to methodological problems. These methodological concerns need to be taken into consideration while interpreting results.

Study design

Paper I-IV used data from the MDCS-CC, which is a prospective cohort study. The prospective study design has its advantages. The information is collected at baseline and the subjects are followed until incidence of outcome of interest or end of the study period. This allows studying of various risk factors and outcomes, and allows establishing a temporal sequence as the risk factors precede the outcome. This type of study design also reduces the risk of recall bias. The study population included is a sample of the total population. The generally longer follow-up period of prospective cohort studies also allows to study diseases with longer latency period such as diabetes.

For **paper V**, cross-sectional data from the SCAPIS cohort was used. A major limitation of the cross-sectional study design is the inability to draw any firm conclusions regarding potential causal relationships.

Internal and External validity

The aim of epidemiological studies is to measure the association between exposure and outcome. It has to be assessed whether or not the observed results are true. Internal validity relates to how well a study has been conducted (142). Therefore, care is taken in epidemiological studies to have internal validity to make valid conclusions from the findings.

External validity relates to how applicable the findings are to other populations, also known as generalisability. For a study to have external validity, it needs to be valid internally. In order to have internal validity, three alternative explanations for the observed association need to be ruled out: 1) Bias, 2) Confounding and 3) Random error or chance.

Bias

Bias in epidemiological studies is a systematic error that results in the observed measure of association to deviate from the true value. Bias can weaken an association, exaggerate it, or reverse its direction (142, 143). It can be introduced during the design or implementation stage of a study and cannot be resolved later.

The two main types of bias are; selection bias and information bias.

Selection bias

Selection bias is a systemic error when the population included in the study is not representative of the target population. In cohort studies, selection bias can occur as a result of loss-to-follow-up and non-response (144). Non-response bias happens when

the participants in the study differ from the non-participants. The participation rate in MDCS-CC was around 71%, which is high. In case of low response from a study, it is essential to take into consideration the differences between the participants and non-participants. The characteristics of the subjects in MDCS-CC and those who did not participate has been remarked (101). Moreover, the response rate for the MDCS-CC re-examination was also quite high (76%). The overall participation rate for SCAPIS was around 53% in Malmö and 58% in Linköping.

Information bias

Information bias can arise as a result of misclassification of exposure and outcome.

Exposure misclassification: The circulating biomarkers were measured once at baseline, and blood samples were frozen and stored at -80°C. The long-term stability of proteins in these samples is not known. However, any minor loss of protein over time, in our opinion, would not affect the positive association identified from the results. Rather, the true effect in the study population would be underestimated. Measurements of arterial stiffness for **paper III and V** were carried out under standard protocol making the possibility of measurement errors low. Moreover, one year reliability analysis was done for c-f PWV for paper **V**.

Outcome misclassification: For **papers I, III and IV**, the main outcome of interest was diabetes. Diabetes, particularly type 2 diabetes, does not have an acute onset and can go undetected for many years. Information regarding incident diabetes during follow-up was retrieved by linkages to several local and national registers, thus increasing the chances to capture all cases. However, it is still likely that some of the cases will have gone undetected, resulting in misclassification. This misclassification, however, would be non-differential, and would lead to attenuation of the observed association. In the MDCS-CC, complete information to distinguish diabetes types was not available. Because the study population included older adults, and because prevalent cases of diabetes were excluded, it is very likely that incident cases were type 2 diabetes.

Additionally, for **paper III**, all individuals with a diagnosis of diabetes according to national or local registers prior to the date of c-f PWV measurements in 2007–2012 were carefully excluded. This was done, as there was delay of an average of 261 days after the first visit in the 2007–2012 examination for logistic reasons.

Survival bias

We recognise that it can be rightfully argued that the aged population of MDCS-CC can be a result of survival bias. Moreover, the subjects also have more use of

pharmacological treatment. However, the aged population would also be at higher risk for cardiometabolic events.

Confounding:

In order for a variable to be a confounder, it should meet the following criteria:

1. It should be associated with the exposure
2. It should be associated with the outcome
3. It should not be an intermediate step in the causal pathway between exposure and outcome.

The relationship between exposure, outcome and confounder is presented schematically in **Figure 14.**

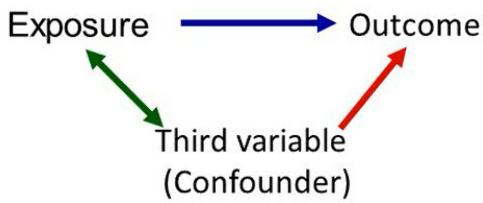

Figure 14:
Relationship between confounder, exposure and outcome

It is crucial that confounding variables in a study be identified, and controlled for as they distort the true association between an exposure and an outcome. Depending on the direction of the associations of the confounder in relation to the exposure and outcome, the true association can be either overestimated (known as positive confounding; when the observed association is biased away from the null) or underestimated (known as negative confounding; when the observed association is biased toward the null).

Generally, it is suggested to select confounders based on a prior knowledge rather than stepwise regression (145). Potential confounders can also be identified by using a diagrammatic representation connecting different variables, the exposure and the outcome. Directed Acyclic Graphs (DAGs) are a graphical tool that can be utilised to visually represent and understand the relationship between exposure, outcome and various confounding variables. The DAGs allow for understanding the concept of causation, confounding, and bias. Visually, we can assess the relationship between the different variables, exposure and outcome, and identify whether a certain variable is a

confounder, a collider or an intermediate variable. A collider is a common effect of both the exposure and outcome and should not be adjusted for. An intermediate variable is identified as on the causal pathway between the exposure and outcome, and also should not be adjusted for (146).

There are various strategies for minimizing confounding, which can be applied at either the design phase, analysis phase or both. At the design stage, this can be done by randomisation, restriction and matching. At the analysis stage, this can be carried out by stratification, matched analysis or adjusting.

The randomisation technique involves the random distribution of individuals to exposure categories. This allows for the distribution of known and unknown confounding variables to be similar for the groups being compared. However, this can be used only in experimental study design. Restriction refers to restricting the analysis in the study to a group of subjects without the confounder. However, restriction can lead to loss of power and generalisability. It can also be difficult to carry out when many confounders are present. Matching is another way to control for confounding whereby investigators identify groups of subjects within a study population who are the same with respect to a confounder of interest. This is often carried out in case-control studies.

At the analysis stage, one way to handle confounding is to stratify the subjects by the confounder of interest. The disadvantage of stratification is again reduction in sample size and statistical power, and the inability to deal with multiple confounding factors simultaneously (147). Another way is to include the confounders in the regression models, and adjust for the effect of confounding.

Confounding can persist even after potential confounders have been adjusted for. There can be several reasons for this residual confounding. For instance, confounders cannot be adjusted for because they were not measured during the process of data collection. In other instances, residual confounding can be introduced when there is error in measurements of variables (148).

Adjustments for potential confounders were carried out in all papers included in the thesis, and have been described in detail in the methods section. Confounders were identified based on review of the existing literature. Additionally, in all papers using c-f PWV, adjustments were done for heart rate and MAP. In **paper III**, in the sub-analysis we further adjusted for additional classical risk factors. This was done due to fewer events per variable in the model. It has been suggested that a minimum of 10 outcome events should be present per predictor variable in a regression model (149). However, this rule has been said to be too conservative, and often it has been acceptable to have less than 10 events per covariate in analyses, especially while conducting sensitivity analyses (150). For **paper V**, potential confounders were evaluated using a DAG.

Random error or chance:

Random error is the other type of error that can lead to difference between the observed and the true estimate. The impact of random error decreases with increased study size, which is documented by lower p-values and narrower CIs. The effect of random error and random sampling variability on the study results can be reduced with a large sample size. In **papers I-V,** a significance level of <0.05 was thought to be adequate for exposure-outcomes situations. It indicates strong evidence against the null hypothesis, as there is less than a 5% probability that the null is correct.

Effect modification or interaction:

An interaction in epidemiological studies occurs when the impact of an exposure on an outcome is changed by the value of a third variable. As with confounding, stratification can be used to explore effect modification further. By stratifying for the interaction variable, the effect of risk factor on the outcome at the different levels of the interaction variable can be observed. Effect modification was tested in all the papers.

Clinical perspective

This thesis has explored the association between known and new biomarkers, and cardiometabolic diseases. The results are of interest because of several reasons.

Firstly, use of biomarkers can potentially be helpful in early risk stratification and allow to re-classify individuals from a low risk category to intermediate or high risk. Secondly, they provide basis for probable pathophysiological speculations. The findings of this thesis are important from a mechanistic point of view. In **papers I and II**, association of inflammatory markers with risk of diabetes and arterial stiffness provides support that inflammation plays a role in cardiometabolic disease progression. In **paper III**, association of increased c-f PWV with increased risk of diabetes provides a novel view of the relationship between arterial stiffness and diabetes. Whereas high glucose levels have been found to be responsible for accelerated stiffening of arteries, the data from **paper III** provokes one to speculate perturbed flow as a possible pathological mechanism for diabetes. In **paper IV**, association of HER2/ ErbB2 with risk of diabetes is a novel and interesting finding, and raises the question whether there is value of exploring an oncogenic marker, which is already established in clinical use. In **paper V**, the association of higher c-f PWV with higher CACS adds value to the use of this marker for identifying those at high risk in asymptomatic individuals.

Conclusions

The thesis examined the associations between arterial stiffness, diabetes, measures of atherosclerosis and inflammatory markers and proteomics, using two large population based cohorts. Based on our findings the following important conclusions can be made:

1. Acute-phase proteins orosomucoid, haptoglobin and CRP are associated with an increased risk of incidence of diabetes. However, after additional adjustment for fasting glucose levels, the association remains significant only for CRP.
2. Elevated plasma levels of alpha-1-antitrypsin, C3 and CRP are independently associated with arterial stiffness, indicating the role of inflammatory dysregulation in arteriosclerosis.
3. Increased arterial stiffness, as determined by c-f PWV, is associated with increased risk of incident diabetes, independent of established risk factors. These results suggest that increased arterial stiffness is an early detectable risk marker for future risk of diabetes, and could be of importance to understand the pathophysiology.
4. Elevated levels of HER2/ErbB2 are associated with increased risk of diabetes. These findings indicate a potentially additional role of HER2/ErbB2 beyond carcinogenesis, and is interesting from a mechanistic and therapeutic point of view.
5. Increased arterial stiffness is positively associated with increasing CACS levels, a marker of sub-clinical atherosclerosis.

Future perspectives

Despite recent advancements in health care, cardiometabolic diseases remain the major cause of mortality and morbidity in the world. Current guidelines for prevention and treatment primarily focus on traditional risk factors such as high blood pressure, cholesterol, smoking and obesity. In the past decade, there has been great interests in the role of arterial stiffness. At present, PWV is not measured routinely in clinics. But this may change in the future. One example of PWV being used is in the screening projects for hypertension by measuring BP in pharmacies in Austria, with the aim to assess vascular age using aortic PWV alongside with BP (151). It should be noted that even though the clinical utility of PWV as a routine examination is yet to be established, the role of vascular ageing in relation to cardiovascular health is well known. In general, clinicians and researchers understand the importance of vascular ageing mirroring biological ageing.

Another aspect of clinical interest is that arterial stiffness can be modified and, therefore, presents an opportunity for targeted treatment approach. Lifestyle changes have shown to decrease arterial stiffness (152). Additionally, certain anti-hypertensive drugs have also shown to play a role in lowering the arterial stiffness (153). In France, the promising SPARTE (Strategy for Preventing Cardiovascular and Renal Events Based on Arterial Stiffness) trial has been initiated with the objective to reduce arterial stiffness by implementing pharmacological interventions (154). Therefore, in the future PWV could be a useful tool for pharmacological and lifestyle modification treatment. Assessing this clinical potential alongside with standardising measurement of PWV would be a step towards incorporating it in clinical practice. The association of arterial stiffness with increased risk of diabetes is an important finding. Moreover, association of increased PWV with CACS highlights the role of arterial stiffness as a risk marker.

In this thesis, biomarkers were analysed to elucidate disease mechanisms at the population level. Inflammation has been known to play a role in cardiometabolic disease, yet is still less characterized than other established risk markers. A need to find the potential use of this association is warranted. HER2/ErbB2 is already a well-established marker in oncology. This additional role of a marker that is already in clinical use can be beneficial. Moreover, the use of proteomics is also a step towards

precision medicine. Although, we still have far to go in this regard but epidemiological studies such as these pave the way to explore pathophysiological pathways.

Epidemiological studies are limited in the fact that they do not establish causality. However, these findings are hypotheses generating. They create a rationale for further exploratory studies, and give a clue that it will be worthwhile to explore associated molecular mechanism, provided they make biological sense. Further studies exploring genetic variants and looking to explore causality would be the next step.

Populärvetenskaplig sammanfattning

Kan nya biomarkörer vara en varningssignal för hjärtkärlsjukdom och diabetes?

Förekomsten av kardiometabola sjukdomar (d.v.s, hjärtkärlsjukdom och diabetes) ökar i världen och utgör ett tydligt globalt hälsoproblem. Kardiometabola sjukdomar och medföljande komplikationer har en djupgående inverkan på livskvalitet och medför en betydande sjukdomsbörda och ekonomisk börda för samhället. Därmed finns ett stort behov av att förstå dessa sjukdomar bättre.

Det finns många viktiga riskfaktorer som påverkar hjärtkärlsjukdom och diabetes. Några av dem är väl kända, såsom rökning, högt blodtryck, fetma, ohälsosamma matvanor och låg fysisk aktivitet. Även om mycket forskning har utförts på området, så är de bakomliggande mekanismerna avseende hjärtkärlsjukdomar och diabetes fortfarande inte helt klarlagda. Det är här kartläggning av nya biomarkörer kan vara till hjälp. Att identifiera nya biomarkörer kan hjälpa oss förstå de underliggande sjukdomsframkallande mekanismerna. Användning av biomarkörer är också till nytta för att identifiera individer med ökad risk för sjukdom i ett tidigt skede och därmed minska deras komplikationer.

När vi åldras så uppstår förändringar i kärlväggen. Våra blodkärl blir stelare, framförallt de större artärerna. Åldrandet leder till förlust av elasticitet på grund av minskat kollagen, som i sin tur leder till stelare artärer. Det är viktigt att klargöra här att artärstyvhet är en skild process från åderförkalkning (ateroskleros), vilket är resultatet av fettavlagringar i artärerna. Artärstyvhet kan mätas på olika sätt. Den vanligaste metoden är att mäta hur snabbt en pulsvåg färdas genom det arteriella trädet, även känd som pulsvågshastighet, uppmätt i meter per sekund. Ju stelare artärer, desto högre pulsvågshastighet. Både artärstyvhet och ateroskleros kan leda till hjärtkärlsjukdom.

Syftet med denna avhandling var att utforska nya biomarkörer relaterade till kardiometabola sjukdomar. I synnerhet så undersökte vi sambandet mellan inflammatoriska markörer, proteiner och artärstyvhet med ökad risk för diabetes. Vidare så undersökte vi sambandet mellan inflammatoriska markörer och artärstyvhet. Vi undersökte också förhållandet mellan artärstyvhet och risken för att ha ett högt kranskärlskalciumsvärde, vilket är ett mått på subklinisk ateroskleros. För att besvara dessa frågor så undersökte vi dessa samband i två stora svenska populationsbaserade kohorter.

I studie 1 och studie 2 undersökte vi sambandet mellan inflammationsmarkörer uppmätta i blodet och förekomsten av diabetes och artärstyvhet. Studie 1 visade att inflammationsmarkören CRP var associerad med en ökad risk för diabetes. I studie 2, när inflammationsmarkörerna utforskades i förhållande till artärstyvhet, så var förhöjda nivåer av alfa-1-antitrypsin, C3 och CRP associerade med en högre artärstyvhet. Vidare så visade resultaten att individer med diabetes hade högre artärstyvhet och att sambandet inte kunde förklaras av förhöjda inflammationsproteiner.

Baserat på resultaten från dessa studier så ville vi i studie 3 utforska sambandet mellan en högre artärstyvhet och ökad risk för diabetes. Det är välkänt att diabetes medför en snabbare utveckling av artärstyvhet, vilket förklarar de vaskulära komplikationer som uppstår vid diabetes. Vi ville dock undersöka om detta samband var dubbelriktat. Vi fann att högre artärstyvhet var kopplat till en ökad risk för diabetes.

Studie 4 undersökte sambandet mellan ett cancerrelaterat protein och risken för diabetes. Vi fann att ett protein, HER2/ErbB2, som är en välkänd markör för bröstcancer, var associerat med en ökad risk för diabetes. Denna upptäckt är intressant eftersom det visar att en markör som redan används för bröstcancer även kan användas till att förutsäga diabetes.

Slutligen så undersökte vi i studie 5 sambandet mellan artärstyvhet och en markör för subklinisk ateroskleros, kranskärlskalciumvärde, för att utforska pulsvågshastighetens roll i förhållande till vaskulär hälsa. Resultaten tyder på att högre artärstyvhet är kopplad till risken för ett högre kranskärlskalciumvärde.

Sammanfattningsvis visade vi i denna avhandling att:

- Inflammatoriska markörer är associerade med ökad risk för diabetes och högre artärstyvhet.
- Högre artärstyvhet är associerad med ökad risk för diabetes och subklinisk ateroskleros.
- HER2/ErbB2, förutom sin roll inom cancer, kan hjälpa oss att identifiera individer med förhöjd risk att utveckla diabetes senare i livet.

Vad detta betyder är att forskning om dessa nya biomarkörer skulle kunna ge en tydligare bild av bakomliggande mekanismer. Våra upptäckter tyder på att de undersökta biomarkörerna kan ha klinisk betydelse och att de bör utforskas vidare. Kunskapen kan vara till hjälp för kliniker och forskare att förstå bakomliggande mekanismer till dessa sjukdomar och att belysa hur de är sammankopplade. Detta kan i sin tur förbättra identifieringen av individer med förhöjd risk i ett tidigt skede och därmed ge möjlighet att förändra sjukdomsförloppet

Popular Science Summary

Can new biomarkers be the warning signal for cardiovascular disease and diabetes?

The prevalence of cardiometabolic diseases is on the rise in the world, and is clearly a global health problem. Cardiometabolic diseases and the associated complications have a profound effect on the quality of life, and impose a substantial health and economic burden on society. Therefore, there is a great need to understand these diseases better.

There are many important risk factors that have an impact on cardiovascular disease and diabetes. Some of them are well known such as smoking, high blood pressure, obesity, poor diet, and lack of physical activity. Although a lot of research has been done in this regard, the underlying mechanisms of cardiovascular diseases and diabetes are still not fully understood. This is where mapping of new biomarkers can be helpful. Identifying new biomarkers can aid us to understand the underlying pathophysiological mechanisms. Use of biomarkers is also useful to identify those at high risk early on in the course of the disease and hence reduce the accompanying complications.

As we grow old, changes occur in the vessel wall. Our blood vessels become stiffer, especially the large arteries. Aging leads to loss of elasticity due to decreased elastin that then result in stiffer arteries. It is important to clarify here that the process of arterial stiffness is separate from the process of atherosclerosis, which results from fatty deposits inside the arteries. Arterial stiffness can be measured in different ways. The most common method is to measure how fast the pulse wave travels through the arterial tree, that is, pulse wave velocity measured in meter/second. The stiffer the arteries, the higher the pulse wave velocity. Both arterial stiffness and atherosclerosis lead to cardiovascular diseases.

The purpose of this thesis was to investigate new biomarkers in relation to cardiometabolic diseases. In particular, we investigated the association of inflammatory markers, proteins and arterial stiffness with increased risk of diabetes. Additionally, we explored the association between inflammatory markers and arterial stiffness. We also examined the relation between arterial stiffness with risk of having high coronary artery calcium score, which is a measure of sub-clinical atherosclerosis. To answer these questions, we explored these associations using two large population based Swedish cohorts.

In Study 1 and 2, we investigated the association of markers of inflammation measured in the blood with incidence of diabetes and higher arterial stiffness. Study 1 showed

that the inflammatory marker CRP was associated with increased risk of diabetes. In Study 2, when the inflammatory markers were investigated in relations to arterial stiffness, elevated levels of Alpha-1-antitrypsin, C3 and CRP were found to be associated with arterial stiffness. Additionally, the results showed that individuals with diabetes had higher arterial stiffness, and raised inflammatory proteins did not explain this relationship.

Based on the findings of these two studies, we wanted to explore the association between higher arterial stiffness and increased risk of diabetes in Study 3. It is well known that diabetes results in the accelerated process of arterial stiffness, and this explains the vascular complications seen in diabetes. However, we wanted to investigate if this relationship was bidirectional. We found that higher arterial stiffness was associated with higher risk of diabetes.

In Study 4, we examined the association between proteins related with cancer and risk of diabetes. We found that one protein, HER2/ ErbB2, which is a well-known marker for breast cancer, was associated with higher risk of diabetes. This finding is interesting as it shows that a marker, which is already in use for breast cancer, can be used to predict diabetes too.

Lastly, in Study 5 we explored the association between arterial stiffness and a marker of sub-clinical atherosclerosis, i.e. coronary artery calcium score, to see the role of pulse wave velocity in vascular health. The findings suggest that higher arterial stiffness is linked with risk of having higher coronary artery calcium score.

In summary, in this thesis we showed that:

- Inflammatory markers are associated with increased risk of diabetes and higher arterial stiffness.
- Higher arterial stiffness is associated with increased risk of diabetes and sub-clinical atherosclerosis.
- HER2/ErbB2, besides its role in cancer, can help us to identify individuals at high risk of developing diabetes later in life.

What this means is that study of these new biomarkers could help provide a better picture of underlying mechanisms of cardiovascular disease and diabetes. These findings suggest that the studied biomarkers may have clinical potential and should be explored further. This knowledge can be helpful for clinicians and researchers to understand the mechanisms behind these diseases, and shed light on how they are connected. This can, in turn, improve identifying at risk individuals early, and thus provide opportunity to change course of the disease.

Acknowledgements

From the time when I began my PhD to this day when I have arrived at this final page of the thesis, penning down the acknowledgements, many people have become part of this amazing journey. This is not only a research journey of four years, but also four years of life filled with interactions and meeting different people, and growing as a person. I would like to express my deepest gratitude to everyone who has been there.

First of all, I would like to thank my main supervisor **Gunnar Engström**. Thank you for giving me this opportunity and for believing in me. I hope you know how much this means to me, and I will always be grateful for that. Thank you for all the knowledgeable discussions, for being so supportive and always available. No matter how worried I would be about something, I would come to your office and our discussion would put me at ease. Thank you for teaching me about science, integrity and hard work. I have been very lucky to have you as a supervisor because not only are you an excellent teacher but also a remarkable person.

My co-supervisor, **Yan Borné**, you were the first person I met when I started my internship in this group. Thank you for all your support, your feedback and guidance over the years, and for our discussions about work and life.

Peter Nilsson, thank you for always sharing your knowledge and the latest information. Thank you for your support on all the arterial stiffness projects, your valued feedback, your recommendations for courses and for being just an email away when I had questions.

Suneela Zaigham, thank you for being a great colleague and friend. I cannot express enough how much I appreciate our discussions on statistics, knowledge and life. Thank you for being such a huge support, for wanting the best for me, for just being there next to me in silence when I needed that, and above all thank you for making me a better human being.

Margaretha Persson, thank you for your feedback on projects, for being so helpful with my queries and sharing your knowledge on the cohorts, and for being a great office mate!

Linda Johnson, thank you for your feedback as a co-author and our discussions over the years. You are an inspiration, and so easy to talk to! It has been a pleasure getting to know you.

John Berntsson, thank you always for your encouragement and your calming advice, and your help with my thesis in the last days. I have truly enjoyed and learnt from our discussions.

Martin Söderholm, thank you for your input as a co-author on the stroke papers, and for your advice and discussions whenever you come by. You have always been so helpful.

Jingxue Pan, thank you for being supportive, and for your wonderful company in Pisa!

Jun, Xue, Edith and other members of the **Cardiovascular Epidemiology group**, former and present, thank you for being great colleagues and for making this a wonderful place to work.

Anders Gottsäter and **Amra Jujic**, thank you for being excellent opponents at my halftime, and for all your constructive feedback and encouragement.

Olle Melander, Marju Orho-Melander, Jan Nilsson, Mikael Gottsäter, Jan E Engvall, Carl J Östgren, and **Bo Hedbald**, thank you all for your feedback and input as co-authors, and for being great peers.

Gerd Östling and **Cecilia Kennbäck**, thank you for all your help with my questions related to arterial stiffness, for your input as co-authors and for taking time out to come to my half time!

Maha Suliman, you have been and always will be my family here in Sweden. I am so grateful to have you in my life! Thank you for being such a big support and encouragement, and for celebrating Eid, and all the milestones of this PhD journey with me.

Kjell Olsson, from writing our Master thesis on the balcony of CRC to writing this PhD thesis, thank you for always being there every step of the way, for all your support, patience, and for always believing in me.

Tobias Herder, our weekly fika meetings at CRC would be something I would look forward to every week! I am very grateful for this "us" time during my PhD. Thank you for making time for me and checking in to see how I am doing.

Hanne Isnaes, thank you for being such a wonderful friend, for checking in how I am doing and for our falafel lunch dates whenever possible.

Madelene Nordström, thank you for always being so supportive and for your company, especially in travels. It has always been fun!

Laura and Doreen, thank you for keeping in touch over the years, for all your support, and for making me feel so welcomed whenever I came to visit.

Joana Alves Dias, thank you for being a friendly face in the office corridor from the very beginning, and later on being such a supportive and kind friend. Thank you for all your advice, encouragement and our walks in the park!

Esther González-Padilla, it has been such a pleasure getting to know you as a friend, and later as a neighbour. Thank you for being so caring, kind and supportive, and for being there.

Stanley Teleka, thank you for all your support, advice and your help during this PhD. You are such a wise and kind person who believes in sharing knowledge. I am especially grateful to you for taking time out to prepare me for my half time!

Bushra Shahida, thank you for being such a caring and generous friend. You have this ability to quietly care about all the people around you and fulfil their needs. Thank you for knowing the importance of a good cup of tea, and for always sharing your tea stash and chocolates with me! Thank you for always caring for me. I am so lucky to have met you.

Tania Singh, when I first met you, I knew I wanted to get to know you more. You are such a strong, brave person and such an inspiration! Thank you for being so thoughtful and caring, and for taking time out to give me feedback on my work.

Ruchi Jain, thank you for always being such a wonderful friend, for your feedback on my work, and for opening up your home to my family and myself. Thank you for being so generous, and for always letting me know that I am very welcome at your home anytime.

Gad Hatem, thank you for trying to make me more cultured by making me listen to classical music. **Pardeep Bompada**, thank you for your help with questions during the thesis writing process.

Njainday, Gaurav, Mansi, Puja, and other friends at CRC, thank you for being such good and supportive company, and for coming to have lunch with me and keeping me company whenever you saw me sitting alone.

Anette Saltin, Claes Moreau, and **Anton Lägerback**, thank you for your prompt help with all my queries and all sorts of administrative work.

Julia, thank you for always stopping by to say hi and to see how my day is going. It is always a pleasure to see you in the mornings. Thank you for putting up with my poor Swedish-speaking skills too!

None of this work could have been possible without **the participants in the MDCS and SCAPIS studies.** Thank you for your time and contribution to research. I would also like to express my appreciation to the **Staff at the Clinical Research Unit at the Department of Internal Medicine, SUS, Malmö** and the **Staff at the Radiology Department who performed CT examinations in SCAPIS,** for their continuous extraordinary work with collecting data for our population studies.

Last but not least, my utmost gratitude to my family, who are my everything. **Mom**, thank you for always, always believing in me, even when I could not believe in myself, and for always making me think I could do whatever I want. **Dad**, thank you for being my biggest support, for always encouraging me and for being proud of me. I would be nowhere without the support of you two. Thank you for all your prayers. Your blessings are my greatest strength and source of peace!

To my siblings, thank you for being my best friends!

My sister, **Hina,** for always being my cheerleader and for always making me see the silver lining. Your strength has always inspired me. Thank you for being my pillar of strength and for always being there for me.

My brother, **Hassan**, being in the same medical field, I had someone in the family I could talk to about things that no one else would get. You have always been there to help, support and encourage me no matter what you would be dealing with yourself. Thank you for being my listener, advisor and counsellor.

Finally, my youngest sibling, **Abdul Rasheed**, thank you for thinking that what your *baji* does is smart, and for being proud of me. Thank you for always being my support and encouragement. And I hope someday you will be able to get through this book I wrote!

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Printed by Media-Tryck, Lund 2020 NORDIC SWAN ECOLABEL 3041 0903

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FACULTY OF MEDICINE

Department of Clinical Sciences

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2020:99
ISBN 978-91-7619-961-9
ISSN 1652-8220