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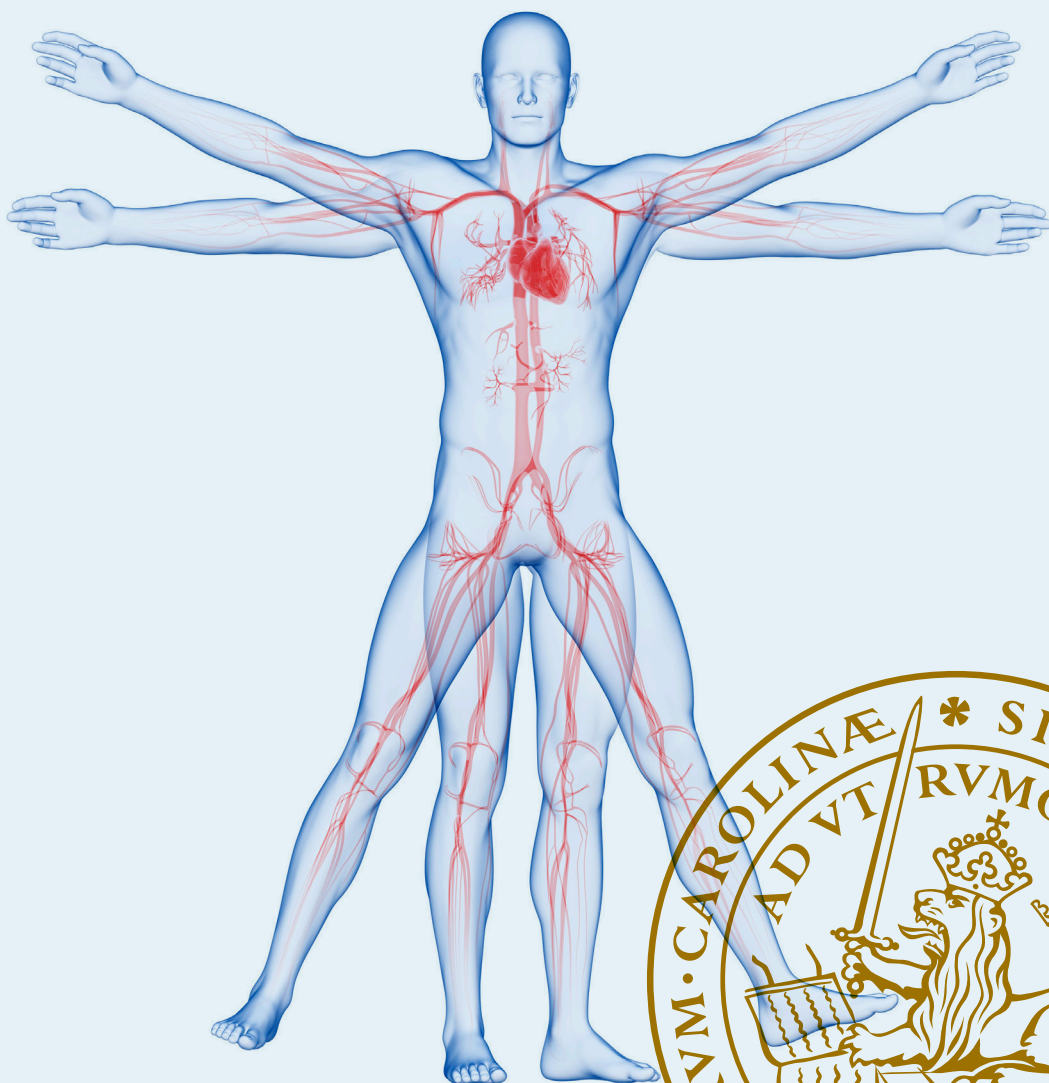
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Epidemiological, mechanistic and genetic aspects of vascular ageing and arterial stiffness in the population

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DEPARTMENT OF CLINICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY 2017



Epidemiological, mechanistic and genetic aspects of vascular ageing and arterial stiffness in the population

Mikael Gottsäter



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DOCTORAL DISSERTATION

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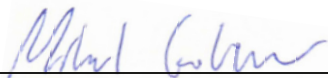
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Title and subtitle: Epidemiological, mechanistic and genetic aspects of vascular ageing and arterial stiffness in the population		
<p>Abstract</p> <p>The core feature of vascular ageing is the age-associated stiffening of the large, elastic arteries, or arteriosclerosis. This results in a diminished volume-buffering function and is therefore central for the increase in systolic blood pressure and pulse pressure seen with advancing age. Since there are considerable individual differences regarding the rate of vascular ageing, the aim was to describe vascular ageing and its relation to hemodynamic, circulating, morphological and genetic markers using cross-sectional and longitudinal data.</p> <p>This thesis is based on epidemiological data from the Malmö Diet and Cancer Study, a population-based cohort from the city of Malmö, Sweden.</p> <p>In Paper 1, adrenomedullin (ADM), a vasoactive peptide mainly produced by endothelial cells, was investigated. The results showed that ADM was positively associated with brachial pulse pressure and both carotid intima-media thickness and atherosclerotic plaques in adjusted models. This suggests a role for ADM in early hemodynamic pathophysiology related to arteriosclerosis and atherosclerosis.</p> <p>In Paper 2 and Paper 3, predictive and cross-sectional associations between arterial stiffness and cardiovascular risk markers were investigated. In Paper 2, the stiffness of the abdominal aorta was assessed by ultrasound while in Paper 3 carotid-femoral pulse wave velocity (c-f PWV) was used, measuring regional arterial stiffness along the carotid-aortic-iliac-femoral arterial segment. In Paper 3, markers of impaired glucose metabolism, dyslipidemia (high triglycerides, low high-lipoprotein cholesterol; HDLc), and waist circumference were all independent, non-hemodynamic, long-term predictors of arterial stiffness, following full adjustment in both sexes. Smoking, low density lipoprotein cholesterol (LDLc), and estimated glomerular filtration rate (eGFR) were not associated with arterial stiffness. These results were partly concurrent with results from Paper 2, the main difference being that insulin resistance and low HDLc were associated with abdominal aortic stiffness among women, but not among men.</p> <p>In Paper 4, Mendelian randomization was used as a method of identifying causal risk factors for arterial stiffness, measured as c-f PWV. Genetic risk scores (GRS) were used as instrumental variables. Arterial stiffness was associated with GRS for fasting plasma glucose (FPG) and type 2 diabetes (T2D). However, in inverse-variance weighted analyzes, significance for FPG β coefficients remained ($p=0.006$) but the relationship between T2D β coefficients was lost ($p=0.88$). GRSs for body mass index, systolic blood pressure, LDLc, HDLc and triglycerides were not associated with arterial stiffness. In conclusion, genetically elevated FPG, but not genetically elevated risk of T2D, was associated with arterial stiffness, suggesting a causal stiffening effect of glycemia on the arterial wall, independently of T2D.</p> <p>To summarize, in a population-based cohort, the risk markers for arteriosclerosis differ from risk markers for atherosclerosis. Results from Mendelian randomization analyses suggest that fasting plasma glucose is a causal risk factor for arteriosclerosis. However, this must be confirmed in future studies including new interventions on hyperglycaemia to improve arteriosclerosis.</p>		
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Content

List of papers	9
Abbreviations	10
Introduction	13
Historical context	13
The arterial wall	14
Histology	14
Elastic properties	15
Arteriosclerosis – stiffening of the elastic arteries	16
Mechanisms of arteriosclerosis	16
Consequences of arteriosclerosis	17
Vascular ageing	18
Impaired glucose metabolism and vascular ageing	19
End-stage renal disease and vascular ageing	19
Differences between arteriosclerosis and atherosclerosis	19
Biomarkers of vascular ageing	20
Blood pressure	20
Local arterial stiffness	21
Pulse wave velocity	22
Carotid intima-media thickness and plaques	23
Adrenomedullin – a circulating biomarker	23
Genetics and arterial stiffness	24
Single nucleotide polymorphisms	24
Genome-wide association studies	24
Genetics of arterial stiffness	24
Mendelian randomization	25
Use of genetic risk score as an instrumental variable	26
Aims	27
Overall aims	27
Specific aims	27
Material and methods	29
Study population	29
The Malmö Diet and Cancer study	29

Paper 1	30
Paper 2	30
Paper 3	30
Paper 4	32
Methods.....	32
Clinical measurements.....	32
Definitions	33
Paper-specific methods	33
Paper 1	33
Paper 2	34
Paper 3	34
Paper 4	35
Statistical methods.....	36
Statistical analysis for Mendelian randomization.....	37
Results	39
Paper 1	39
Paper 2.....	41
Paper 3.....	43
Paper 4.....	48
Discussion.....	51
Markers of vascular ageing	51
Obesity.....	51
Lipids.....	52
Smoking.....	53
Glomerular filtration rate.....	53
Adrenomedullin	54
Diabetes and hyperglycemia.....	55
Markers of local abdominal arterial stiffness	55
Risk factors of vascular ageing	56
Strengths and limitations.....	58
Clinical perspectives	59
Erratum.....	60
Conclusion.....	61
Future perspectives	63
Populärvetenskaplig sammanfattning på svenska	65
Acknowledgements	67
References	69

List of papers

This thesis is based on the following four original papers.

1. **Gottsäter M**, Ford LB, Ostling G, Persson M, Nilsson PM, Melander O. Adrenomedullin is a marker of carotid plaques and intima-media thickness as well as brachial pulse pressure. *J Hypertens*. 2013; 31(10):1959-65.
2. **Gottsäter M**, Länne T, Nilsson PM. Predictive markers of abdominal aortic stiffness measured by echo-tracking in subjects with varying insulin sensitivity. *J Hum Hypertens*. 2014; 28(7):456-60.
3. **Gottsäter M**, Östling G, Persson M, Engström G, Melander O, Nilsson PM. Non-hemodynamic predictors of arterial stiffness after 17 years of follow-up: The Malmö Diet and Cancer study. *J Hypertens*. 2015; 33(5):957-65.
4. **Gottsäter M**, Hindy G, Orho-Melander M, Nilsson PM, Melander O. A genetic risk score for fasting plasma glucose is independently associated with arterial stiffness: A Mendelian randomization study. 2017 (submitted manuscript).

Abbreviations

ADM	Adrenomedullin
BMI	Body mass index
BP	Blood pressure
b-a PWV	Brachial-ankle pulse wave velocity
c-f PWV	Carotid-femoral pulse wave velocity
cIMT	Carotid intima-media thickness
CKD	Chronic kidney disease
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
eGFR	Estimated glomerular filtration rate
ESRD	End-stage renal disease
EVA	Early vascular ageing
FPG	Fasting plasma glucose
GRS	Genetic risk score
GWAS	Genome-wide association study
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IV	Instrumental variable
HDLc	High density lipoprotein cholesterol
HOMA-IR	Homeostatic model assessment of insulin resistance
HR	Heart rate
LDLc	Low density lipoprotein cholesterol
MAP	Mean arterial blood pressure

MDCS	Malmö Diet and Cancer study
MDCS-CV	Cardiovascular arm of the Malmö Diet and Cancer study
MR	Mendelian randomization
MR-proADM	Mid-regional part of pro-adrenomedullin
OGTT	Oral glucose tolerance test
PP	Pulse pressure
PWA	Pulse wave analysis
PWV	Pulse wave velocity
RCT	Randomized controlled trial
SBP	Systolic blood pressure
SMC	Smooth muscle cell
SNP	Single nucleotide polymorphism

Introduction

Historical context

The history of assessing the arterial pulse is ancient. Nearly 5000 years ago, in the Chinese “Yellow Emperor’s classic of internal medicine” Huang Ti (2698-2598 BC) states: *“Hence, if too much salt is used in food, the pulse hardens...”* [1]. Assessment of the pulse has kept an important role in traditional Chinese medicine and some pulse patterns are actually interpretable parameters using modern pulse wave analysis [2]. The insight that ageing is related to changes in our arteries was expressed in the 17th century by the famous British physician Thomas Sydenham (1624-1689 AD) who stated *“A man is as old as his arteries”* [3].

One of the first investigators to scientifically assess the arterial pulse wave was the British physician, Fredrik Akbar Mahoumed (1849-1884). In the 19th century, he conducted experiments on pulse wave analysis, performing tonometric evaluations of the radial artery with a tool of his own design, the Sphygmograph [4]. Furthermore, in 1922, Bramwell and Hill published an important paper for measuring arterial stiffness as carotid-femoral pulse wave velocity (c-f PWV) [5], the gold standard of today [6]. This paper includes descriptions of the mathematical background and assumptions of c-f PWV [5]. However, the new discovery of blood pressure (BP) measurements and their diagnostic value meant that pulse wave analysis and velocity were sparsely used for much of the remaining 20th century [7].

BP measurements as we know them today were first described by the Russian military physician Nikolai Korotkoff (1874-1920) in 1905 [8]. His observation of the sound from the constricted artery made assessment of the diastolic, in addition to systolic blood pressure, possible. The value of BP measurement was soon recognized and included in the routine medical examination [7]. For decades, the field of hypertension came much to focus on diastolic blood pressure (DBP) and a hypertension diagnosis required an elevated DBP. During the 1980s and 1990s, systolic blood pressure (SBP) was increasingly recognized as a risk factor for cardiovascular disease (CVD) [7]. Furthermore, the rise in SBP in advanced age was attributed to stiffening of the large arteries [9]. This led to a renewed interest in the measurement of arterial stiffness and pulse wave analysis [7]. The first paper establishing the role of arterial stiffness for prediction of cardiovascular mortality

was published in 2001 [10] and population-derived reference values for c-f PWV were published in 2010 [11]. The field has grown rapidly and there is now a large number of methods and devices available for assessing an individual's arterial properties, and the concept of vascular ageing, for which arterial stiffness is the core feature, has gained increasing popularity during the last decade [12-14].

The arterial wall

Histology

Histologically, the human large arteries are composed of three different layers. The innermost layer, the *tunica intima*, consists a single cell layer of endothelial cells in direct contact with the blood flow, a thin layer of connective tissue, and an internal elastic lamina [15]. The endothelium is essential to the vascular function as it synthesizes, releases and responds to a large number of vasoactive substances regulating vasoconstriction/dilatation, thrombosis, inflammation and angiogenesis [16]. The medial layer, the *tunica media*, constitutes layers of smooth muscle cells (SMC), elastic fibers rich in elastin supported by stronger, load-bearing collagen fibers [15]. The SMCs are circumferentially arranged in most of the tunica media except for the outermost part where they are longitudinally organized. The outermost layer, the *tunica adventitia* or *tunica externa*, contains connective tissue primarily composed of collagen and is often not distinct from connective tissue outside the artery. Small blood vessels, the *vaso vasorum*, are also located in the tunica adventitia of large arteries, where they provide perfusion for the vascular wall. Within this layer also runs the *nervi vasorum* which is an important structure for regulating vasoconstriction/dilatation by sympathetic and parasympathetic nerve fibers contracting and relaxing the SMCs [16].

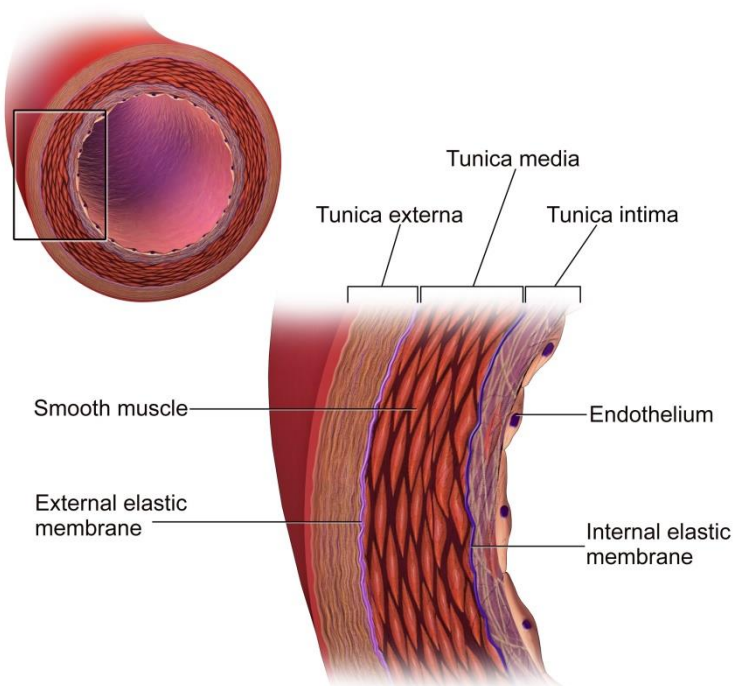


Figure 1. Basic histological structure of the arterial wall. Copyright © Blausen.com staff [17].

Elastic properties

The large, proximal arteries called *elastic arteries* differ histologically from the smaller, more distal, *muscular arteries* [16]. An important difference is that the content of elastin is higher in elastic arteries while the tunica media of the muscular arteries has a higher percentage of SMCs and collagen. However, these changes occur gradually when moving distally from the heart, ergo the most pronounced elastic properties are found in the thoracic aorta [18]. The central arteries, primarily the aorta, expand during systole, accommodating some of the ejected blood which is later expelled into the circulation during arterial recoil in diastole. In a young individual, the aorta dilates around 10% with each heartbeat [19]. This cushioning, volume-buffering effect is known as the *Windkessel* effect and enables a more continuous blood flow to the peripheral circulation and reduces cardiac work [20, 21]. The muscular arteries are more able to contract or relax and thereby regulating the elastic properties of the arterial wall [16]. The elastic properties of elastic arteries, on the other hand, are more dependent on loading pressure. At smaller

distension, elastin is the major load-bearing component of the arterial wall, thus giving it elastic properties [22, 23]. At greater pressures the artery distends and the collagen in the wall limits further extension, increasing the functional wall stiffness.

When the pressure wave generated in systole reaches sites of resistance such as arterial branches, tortuosity or change in arterial diameter, some of its energy is reflected backwards, protecting the peripheral circulation from pulsatile energy [16, 20]. The impedance mismatch between the elastic and muscular arteries is an important contributor to this phenomenon. The reflection wave returns to the heart in diastole thereby increasing diastolic pressure and enhancing cardiac perfusion [20].

Arteriosclerosis – stiffening of the elastic arteries

Mechanisms of arteriosclerosis

Several mechanisms, which are only partially clarified, account for the structural changes occurring in the elastic arteries with increasing age. The stretching and relaxation with every heartbeat gradually damage the structural components of the arterial wall [24]. At 70 beats per minute the arterial wall stretches 30 million times per year, resulting in material fatigue with thinning, fraying and fracturing of the elastic lamellae [24]. In contrast to elastin, the amount of collagen increases, and together with other structural proteins, forms abnormal cross-linkages [25, 26]. This is frequently accompanied by a deposition of calcium in the degenerated elastic fibers and the extra cellular matrix, and is particularly prominent in end-stage renal disease (ESRD) [25, 27]. The stiffening process is also affected by systemic inflammation [28] and metabolic changes such as hyperglycemia, resulting in non-enzymatic glycation of arterial wall proteins [29]. Furthermore, the Angiotensin II type I receptor plays a role in the development of arterial stiffness through promotion of hypertrophy and fibrosis in the arterial wall [30]. These findings are supported by the non-BP dependent lowering effects on arterial stiffness achieved by treatment with angiotensin enzyme conversion inhibitors and angiotensin II receptor blockers [31]. A number of additional mechanisms are also thought to play a role in the stiffening process. These include cellular immune mechanisms, vascular SMC stiffening, and abnormal protein degradation by matrix metalloproteinase-12 [32]. The muscular arteries are not subject to the same high amount of stretching and stiffening as compared to the elastic arteries [33-35].

Consequences of arteriosclerosis

With increasing age, the elastic arteries stiffen leading to a loss of volume-buffering functions and increased pressure wave speed [20]. Also, the reflective wave travels faster in a stiff artery, causing this wave reflection to return while still in systole rather than diastole.

Both the stiffness itself and the early return of wave reflection result in an increased left ventricular (LV) afterload. This leads to cardiac LV hypertrophy, increased oxygen requirements and finally LV failure [15, 36, 37]. LV hypertrophy is shown to increase the systolic to diastolic time ratio, which, together with a decreased diastolic pressure, results in a compromised coronary blood flow during diastole [20]. Together, increased blood demand and capacity reduction of the coronary perfusion predisposes to myocardial ischemia [38].

The stiffening of elastic arteries results in an increased SBP and a decreased DBP, thereby further increasing pulse pressure (PP) [15]. Throughout life, the SBP rises with increasing age while the DBP reaches its peak at age 50-60 years [9]. Arterial stiffness is therefore indeed an important underlying cause of the marked increase in systolic hypertension seen in the elderly population [39]. The stiffness is also associated with an increase in both short and long-term BP variability [40].

A more pulsatile blood flow associated with stiffness of elastic arteries stimulates hypertrophy and increases peripheral resistance, leading to increased mean arterial pressure (MAP). An increase in MAP distends the elastic arteries and exacerbates functional arterial stiffness, thus creating a vicious cycle [13, 23, 41].

The energy of the pulsatile blood flow caused by the arterial stiffness is transmitted to the microcirculation. Attenuation of the stiffness gradient between central and peripheral arteries further aggravates the situation [42]. The brain and the kidneys, organs with high resting blood flows, are especially vulnerable [24, 43]. High pulsatile shear stress dislodges the endothelial cells, leading to thrombosis and micro-infarctions. When the BP exceeds the protective mechanisms of renal autoregulation, glomerular hyperfiltration ensues, leading to glomerulosclerosis and diminished glomerular filtration rate (GFR) [44]. In the brain, high blood flows and transmitted pulse energy are associated with white matter lesions (WML) described as “pulse wave encephalopathy” [45, 46].

In conclusion, arterial stiffness has a number of negative hemodynamic consequences affecting primarily the heart, the kidneys, and the brain. Two meta-analyses have demonstrated arterial stiffness to be an independent predictor of stroke, myocardial infarction, cardiovascular mortality and all-cause mortality [47, 48].

Vascular ageing

Since arteriosclerosis is so dependent on age, the stiffening of the elastic arteries during the course of life is often referred to as vascular ageing. Still, as outlined above, the rate of ageing is dependent on a number of known and unknown factors (further outlined in the “Accelerated vascular ageing” section) and therefore arteriosclerosis is dependent not only on chronological age [12]. In the last decade, the term “Early Vascular Ageing” (EVA) has been coined to describe individuals presenting with advanced arteriosclerosis early in life [12, 49]. However, no exact definition of EVA has yet been established [14]. Even though the core feature of vascular ageing is arteriosclerosis, there are a number of other age-dependent changes occurring in the arteries such as atherosclerosis, aortic dilatation, arterial wall hypertrophy, endothelial dysfunction and small artery remodeling [18, 50, 51]. It is also likely that EVA corresponds to a general ageing process taking place outside the vessels. This reasoning is supported by the fact that arterial stiffness is shown to be a predictor of all-cause mortality [13, 47, 48].

The relationship between arteriosclerosis and hypertension is complex. First, arteriosclerosis, because of the reduced cushioning effect, increases PP [15]. At the same time, high BP is intrinsically associated with arterial stiffness through an increased loading of stiff components in the arterial wall. If the high BP persists, the chronically elevated BP causes damage to the arterial wall [52].

In hypertension there exists a cross-talk between large and small arteries constituting a vicious cycle of BP increase [41]. In one longitudinal study, arterial stiffness was able to predict hypertension, while BP at baseline was not able to predict progression of stiffness [39]. In another study, SBP was associated with an increase in PWV after follow-up [53].

Apart from hypertension, arterial stiffness has also been shown to have an association with: (1) traditional cardiovascular risk factors such as type 1 and 2 diabetes, obesity, ESRD, dyslipidemia and smoking; (2) inflammatory diseases such as inflammatory bowel disease, vasculitis, systemic lupus erythematosus and rheumatoid arthritis; and (3) other factors such as low birth weight and physical activity [6, 54]. However a systematic review from 2009 of cross-sectional studies showed an association between c-f PWV and diabetes in only 52% of the studies [55]. Independent associations between c-f PWV and dyslipidemia, smoking or body mass index (BMI) were found only in a minority of the studies. It should be noted, however, that the review included many smaller studies and selected study populations not representing the general population.

Impaired glucose metabolism and vascular ageing

A large body of evidence supports that arterial stiffness is indeed increased in both type 1 and type 2 diabetes and that it is an early phenomenon occurring before the onset of clinical complications [56]. Furthermore, increased arterial stiffness has been shown to occur already in a pre-diabetic state of impaired glucose metabolism where both impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) have been reported to be associated with stiffer arteries [56-59]. Both type 1 and type 2 diabetes have also been shown to increase the age-dependent speed of arterial stiffening, thereby accelerating the vascular ageing process [56]. Finally, when clinical microvascular and macrovascular complications do emerge, they are accompanied by a further increase in arterial stiffness [56].

Arterial stiffness might be one important factor linking diabetes to the well-known increased cardiovascular risk seen in this condition. However, it is not clear whether the hyperglycemia or the insulin resistance is most important in the pathophysiology [60]. Arterial stiffness has also been shown to be associated with an increasing number of traits of metabolic syndrome [61]. The results for diabetes type 1, on the other hand, support a more prominent role of hyperglycemia in and of itself [56].

End-stage renal disease and vascular ageing

In addition to the negative hemodynamic effects of arterial stiffness on glomerular function, a more severe reduction of GFR leads to an increase in arterial stiffness as seen in patients with ESRD [62]. This is attributed to a disturbed calcium-phosphate balance leading to a calcification of smooth muscle cells and extra-cellular matrix of the arterial wall [25, 27, 62]. The ESRD patient population was one of the first studied populations where the concept of arterial stiffness was implemented and shown to be associated with cardiovascular mortality [63]. The association between arterial stiffness and chronic kidney disease (CKD) stages 2-4 is not clear, as the published results have had conflicting conclusions [64].

Differences between arteriosclerosis and atherosclerosis

Despite often being confused with atherosclerosis, it is important to emphasize that arteriosclerosis and atherosclerosis are two different entities. While arteriosclerosis is a morphological condition found in the tunica media of large elastic arteries, atherosclerosis consists of a focal accumulation of lipids, inflammatory cells and calcium in the tunica intima [51]. These accumulations form plaque that narrows the arterial lumen and obstructs blood flow. A rupture of the atherosclerotic plaque

can lead to thrombosis and vessel occlusion, often manifested as a cardiovascular event such as myocardial infarction or stroke.

Table 1. Key differences between arteriosclerosis and atherosclerosis. Modified from O'Rourke 2015 [51].

	Arteriosclerosis	Atherosclerosis
Important locations	Aorta	Coronary arteries, Carotid arteries
Histological location	Media	Intima
Lumen	Dilatation	Occlusion
Distribution	Diffuse	Focal
Important risk factors	Age and blood pressure	Cholesterol and smoking
Consequence	Loss of cushioning function	Loss of conduit function

Although arteriosclerosis and atherosclerosis often coexist although they differ in many respects. They share several cardiovascular risk factors and the presence of one accelerates the other. Complications including heart failure and stroke are mutual and at advanced stages and in later life the two entities coalesce [24].

Biomarkers of vascular ageing

Blood pressure

As previously described, one important consequence of arteriosclerosis is the increase in SBP and decrease in DBP, resulting in an increased PP [15]. However, since the brachial artery is a muscular artery it is not as affected by arteriosclerosis the same way the elastic arteries are [33-35]. The PP amplification expressed as the ratio between brachial PP and central PP decreases from 1.7 in a population below 20 years old to 1.2 in a population above 80 years old [65]. As a consequence, measurement of brachial BP significantly underestimates the age-associated increase in central PP. Central PP is therefore a better marker of arteriosclerosis than brachial PP and more accurately reflects the impact of BP on the heart, kidneys and brain. In a meta-analysis, measuring central PP compared to brachial PP was marginally, but not significantly ($p= 0.057$) better for predicting cardiovascular events [66].

Basic physiological principles dictate that the mean arterial pressure (MAP) = cardiac output (CO) x systemic vascular resistance (SVR). An increased MAP leads to an increased distension of elastic arteries, thereby increasing PP [23]. However, PP is also directly affected by the SVR. Overall, BP is influenced by several factors other than arteriosclerosis. Therefore, even if they can be used as surrogate markers,

neither peripheral nor central BP represent ideal methods for estimating the degree of arteriosclerosis.

Local arterial stiffness

The compliance of elastic arteries is not homogenous but rather differs markedly even within the aorta where the thoracic aorta is most compliant [18]. The stiffness of a specific arterial segment can be assessed using an ultrasound technique called echo-tracking [6]. During such measurements, the distensibility can be determined by relating changes between the diastolic and systolic arterial lumen diameter/area and BP. Arterial distensibility is defined as the relative change in vessel diameter for a given change in BP. The distensibility (or stiffness, the inverse of distensibility) is calculated directly without using any model or assumptions of the circulation. Several different indices have been used including the β -stiffness index, established for use *in vivo* by Kawasaki et al [67].

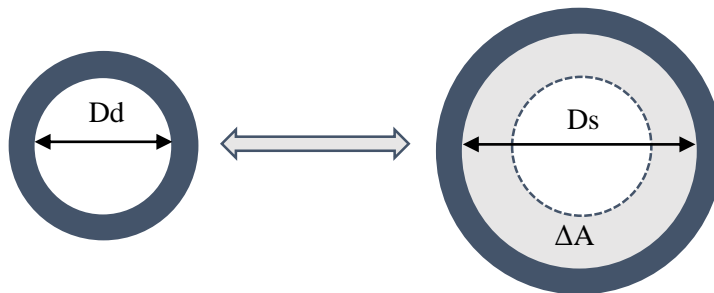


Figure 3. Assessment of local arterial distensibility by relating the pulse pressure to the change in arterial diameter or cross-sectional area. Dd, arterial lumen diameter in diastole; Ds, arterial lumen diameter in systole; ΔA , change in arterial cross-sectional lumen area between systole and diastole.

Pulse wave velocity

Today, pulse wave velocity (PWV) is a generally accepted tissue biomarker of arterial stiffness [68]. The measurement of PWV utilizes the principles of energy propagation where waves travel faster in a rigid tube [6, 69]. Therefore, loss of distensibility results in an increased PWV. The mathematical model linking PWV to arterial distensibility, BP and blood density was described by Bramwell and Hill, who derived their model from the Moens-Korteweg equation [5, 69, 70]. According to the Bramwell-Hill equation, PWV is linked to geometrical changes of the artery by the formula:

$$PWV = \sqrt{\frac{\Delta P \times V}{\Delta V \times p}}$$

where ΔP is change in blood pressure, ΔV is change in blood volume, V is blood volume and p is blood density [69].

The waveform is measured at two sites and generally obtained transcutaneously using various techniques including pressure, distension or Doppler [6]. The transit time, Δt , can be measured between the feet of the two waveforms (known as the foot-to-foot method). The distance between the measuring sites, ΔL , is best measured as the direct distance between the measuring points multiplied by 0.8 to approximate the true arterial distance [71]. PWV is then calculated as $PWV = \Delta L / \Delta t$. When assessing c-f PWV, the waveforms are measured in the right common carotid and right femoral artery and therefore include the entire length of the carotid–aortic–iliac–femoral pathway [6]. The c-f PWV is therefore a measurement of regional arterial stiffness. Currently, c-f PWV has been established as the gold standard method for measuring arterial stiffness according to a consensus document [6]. A c-f PWV value exceeding 10 m/s is considered elevated (provided the distance is calculated as direct distance multiplied by 0.8) [71].

Alternatively, the waveforms can be retrieved from the brachial and ankle regions [72]. Brachial-ankle PWV (b-a PWV) reflects a longer arterial length but includes both elastic and muscular arteries and has weaker evidence for cardiovascular disease prediction [68].

Analysis of the arterial waveform, called pulse wave analysis (PWA), also makes it possible to calculate central BP via transfer functions, and to quantify the pulse pressure amplification (Augmentation Index, AIx) [73].

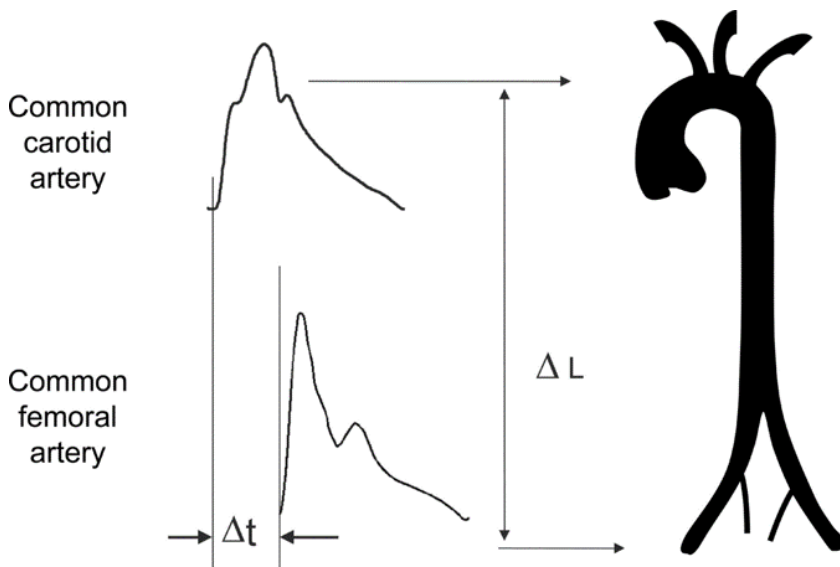


Figure 2. Measurement of carotid-femoral pulse wave velocity using the foot-to-foot method. Laurent S, et al. Eur Heart J. 2006 [6]. With permission from Oxford University Press.

Carotid intima-media thickness and plaques

The thickness of the carotid tunica intima and media as well as the occurrence of plaque can be visualized and measured by ultrasound. Carotid intima-media thickness (cIMT) is most easily measured in the distal carotid artery [74]. Both cIMT and carotid plaque are markers of atherosclerosis but plaque may represent a later stage or a phenotype of atherosclerotic disease other than cIMT. Epidemiological studies have used several different definitions of plaque. According to the Mannheim consensus document, plaque is defined as “focal structures encroaching into the arterial lumen of at least 0.5 mm or 50% of the surrounding IMT value, or demonstrates a thickness >1.5 mm as measured from the intima-lumen interface to the media-adventitia interface” [74].

Adrenomedullin – a circulating biomarker

Adrenomedullin (ADM) is a 52 amino-acid peptide secreted primarily from endothelial cells as a response to different types of cellular strain such as ischemia [75, 76]. It has vasodilator properties by increasing nitric oxide (NO) and decreasing endothelin in the endothelial cells [76]. It also inhibits SMC proliferation. ADM exerts its effects in an autocrine, endocrine and paracrine pattern [77]. Several

studies suggest that ADM is a compensatory hormone promoting natriuresis, diuresis and vasodilation [78-80]. In addition, it has protective properties for the vascular wall [79, 80]. ADM has been shown to predict cardiovascular events, but results from epidemiological studies regarding its associations to atherosclerosis and arteriosclerosis in general populations have been inconclusive [81, 82].

Genetics and arterial stiffness

Single nucleotide polymorphisms

A single nucleotide polymorphism (SNP) is a variation of a single base pair in the human genome occurring in coding or non-coding region. In the 1000 Genomes Project, more than 38 million SNPs with a frequency of more than 1% were identified in the human genome [83]. However, most of them are in linkage disequilibrium (LD) with each other, meaning they are likely to be inherited together [84]. Also, only 12% of SNPs associated with traits are located in, or in tight LD, with protein-coding regions of genes [85]. The remaining 88% of trait-associated SNPs are either found in intergenic regions or non-coding introns.

Genome-wide association studies

A genome-wide association study (GWAS) is a large-scale association study typically testing the relationship between a phenotype (usually a complex trait) and around 1×10^6 SNPs [86]. Because of the impartial approach with multiple testing, the threshold for significance must be very stringent, typically 5×10^{-8} , therefore requiring very large study populations. Also, results from GWASs should be validated in replication studies. In the last decade, GWASs have generated a large number of publications and revolutionized the mapping of genetic influences on complex traits.

Genetics of arterial stiffness

According to results from twin and family studies, the heritability of arterial stiffness is approximately 40% [87, 88]. Despite a moderately high heritability and numerous associations between arterial stiffness and cardiovascular risk markers, GWASs have identified only one genetic variant to be significantly associated with arterial stiffness and results have not been concurrent [89, 90]. A multicenter GWAS of 20,364 individuals from 9 European community-based cohorts has identified one

significant locus in the 3'-*BCL11B* gene desert on chromosome 14 [90]. Another study showed a borderline significant result for the *COL4A1* gene on chromosome 13, coding for Collagen type 4 [89].

Mendelian randomization

Epidemiological studies are subject to many biases including confounding factors and reverse causality, which markedly impairs the ability to demonstrate causal associations [91]. Even if a *risk marker* can be valuable and useful merely by its role in prognosis/prediction, it is not necessarily a *risk factor* involved in the etiology of the disease. To address the issue of causality, randomized controlled trials (RCT) have been used to balance the effect of known and unknown confounders between the groups. However, apart from RCTs being very expensive, they are not always feasible or ethical [92]. Mendelian randomization (MR) is a technique using genetic information to achieve an unbiased detection of causal effects [91]. In analogy with an RCT, individuals are randomly assigned to either a high or a low level of the exposure (marker) depending on whether the individual is a carrier of the risk allele or not. The outcome of interest can then be measured and compared between the groups.

The principle behind MR is that a causal relationship between marker and outcome should be accompanied by the outcome and a genetic variant for the marker [92]. This way, a genetic variant is used as an instrumental variable (IV) benefitting from the random allocation of alleles at conception and the absence of confounding factors or reverse causality. To be used as an IV, three assumptions should be met. The genetic variant should be: (1) unrelated to confounding factors; (2) reliably associated to the marker; and (3) associated to the outcome only through the marker [92]. This is illustrated in Figure 4.

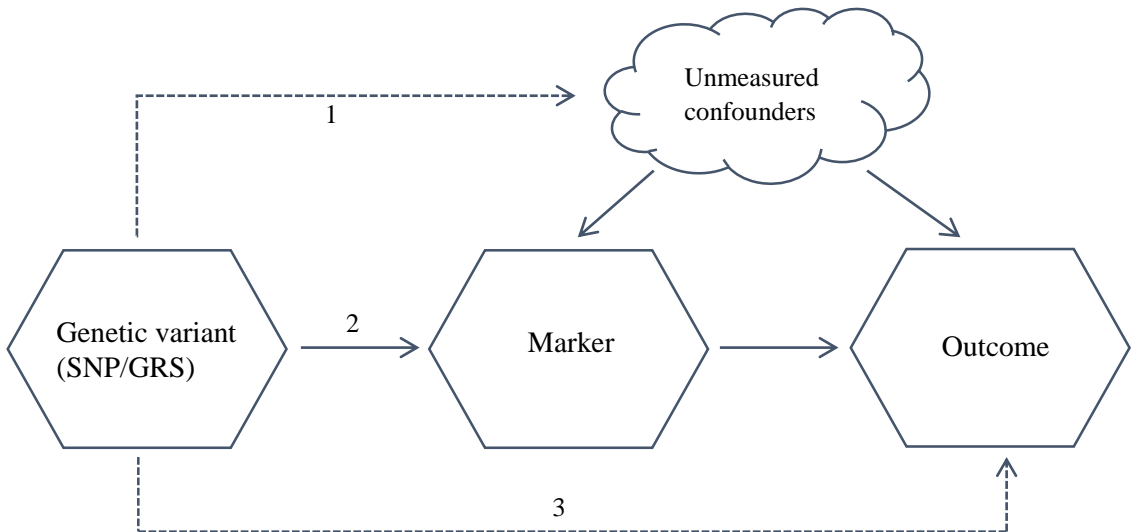


Figure 4. Schematic figure of the use of a genetic variant as an instrumental variable in Mendelian randomization. The genetic variant should be: (1) unrelated to confounding factors; (2) reliably associated to the marker; and (3) associated to the outcome only through the marker. SNP, single nucleotide polymorphism; GRS, genetic risk score.

Use of genetic risk score as an instrumental variable

Most commonly, an SNP is used as an IV in MR. However, the majority of identified SNPs associated with complex traits carry low associated risks and individually account for little heritability [86]. Therefore, a combination of several SNPs known to be associated with a certain trait of interest can be used as an IV in MR [93]. This can be achieved either by combining several SNPs to a single genetic risk score (GRS) or by using a summary statistical method where causal estimates calculated from each SNP are combined in an inverse-variance weighted meta-analysis.

Aims

Overall aims

To describe vascular ageing and its relation to hemodynamic, circulating, morphological and genetic markers.

To use longitudinal data to describe predictive markers of vascular ageing.

The overall aim was to better understand the mechanisms behind development of arterial stiffness. As arterial stiffness is an independent risk factor for cardiovascular disease, the hypothesis was that markers previously implicated in cardiovascular disease are predictors of arterial stiffness.

Specific aims

Paper 1: To investigate the cross-sectional relationships between the circulating marker adrenomedullin and measurements of atherosclerosis and arteriosclerosis.

Paper 2: To explore the cross-sectional and longitudinal relationships between local arterial (abdominal aorta) stiffness and markers of glucose and lipid metabolism, as well as obesity.

Paper 3: To explore the cross-sectional and longitudinal relationships between regional arterial (aortic) stiffness and a series of cardiovascular risk markers including markers of glucose and lipid metabolism, renal function, smoking and obesity.

Paper 4: To use a Mendelian randomization approach to explore potential causal relationships between regional arterial (aortic) stiffness and cardiometabolic risk markers by the use of genetic risk scores of blood pressure, adiposity, impaired glucose metabolism, and dyslipidemia.

Material and methods

Study population

The Malmö Diet and Cancer study

The four papers in this thesis all include individuals from the Malmö Diet and Cancer study (MDCS). The MDCS is a large, population-based prospective cohort from the city of Malmö, Sweden, examined between 1991 and 1996, which aimed to explore the relationship between diet and cancer [94]. Men born 1923-1945 and women born 1923-1950 living in the city of Malmö were invited to participate [95]. The only exclusion criteria were mental disability or inadequate Swedish language skills. Individuals were recruited via public advertisements and personal letters. In all, 28,449 individuals, corresponding to 41% of the eligible population, attended [95]. To investigate the representability of MDCS compared to the population of Malmö, a health survey called the Health Situation in Malmö '94 (HSM:94) was sent out to an age-matched population [95]. The 74.6% participation rate of HSM:94 was substantially higher than in MDCS and allowed for comparison of participants between the both groups. Prevalence rates of smoking, obesity and socio-demographic characteristics were similar in both study groups [95]. However, self-estimated poor well-being was higher in MDCS participants, while cardiovascular and cancer mortality were both considerably lower.

A random sample from MDCS of every second individual was invited to a sub-study including an ultrasound examination of the right carotid artery [96]. In all, 6103 individuals took part in this “Cardiovascular cohort” or “Cardiovascular arm” of the MDCS cohort (MDCS-CV), which was studied between October 1991 and February 1994. Blood samples were not collected at the time of the ultrasound measurement, but at a separate visit: Of the 6103 individuals in the MDCS-CV, 5540 individuals returned for fasting blood sample collection [97].

Between May 2007 and September 2012, a total of 3734 individuals participated in the re-examination of the MDCS-CV [98, 99]. Of the original MDCS-CV population, 2% had emigrated, 17% had died and 19% were not attending due to other reasons (unwillingness, sickness, lack of information in registers) [98]. The

two latter groups were older and, based on variables obtained at baseline, were in many aspects less healthy than the participants in MDCS-CV re-examination [98].

The MDCS-CV and MDCS-CV re-examination were both approved by the Regional Ethics Review Board, Lund, Sweden (Baseline ID LU-5190, Re-examination ID 532-2006).

Paper 1

Paper 1 is a cross sectional analysis of MDCS-CV using analyses of mid-regional part of pro-adrenomedullin (MR-proADM) from frozen plasma collected at baseline. From the 5540 individuals in the MDCS-CV with available blood samples, 4924 individuals (mean age 58 years, 40% men) were included in the study population. The rest were excluded because of missing data. Of those, measurements of cIMT and a six-graded plaque score were available in 4888 and 3384 individuals, respectively. During the initial phase of the study period, the carotid plaques were graded according to another scale explaining the lower number of individuals with available six-graded plaque score.

Paper 2

Between 1999 and 2000, 909 subjects from the MDCS-CV were re-examined for risk factors associated with insulin resistance [100]. In order to study the effects of impaired glucose metabolism these participants were selected according to degree of insulin sensitivity as estimated by the homeostatic model assessment of insulin resistance (HOMA-IR) levels [101]. In total, 15% were sampled from each of the lowest two quartiles of the HOMA-IR distribution, 30% from the third quartile and 40% were sampled from the subjects with baseline HOMA-IR in the fourth quartile [100]. Thereby, individuals with insulin resistance were deliberately over-represented. Of the 909 subjects in this, so called, HOMA cohort, 349 were randomly selected to an ultrasonographic investigation of the abdominal aorta. From the 349 individuals examined with ultrasonography, complete data were available from 335 individuals (mean age 64 years, 42% men), which constituted the study population in Paper 2.

Paper 3

Paper 3 consists of baseline MDCS-CV and MDCS-CV re-examination data. Of the 3734 individuals included in the MDCS-CV re-examination, 3056 individuals underwent successful measurement of arterial stiffness with c-f PWV. Of the 678

individuals with missing c-f PWV data, 387 individuals were invited but did not participate. The rest, 291 individuals, had missing data due to atrial fibrillation or other arrhythmias precluding the c-f PWV measurement. Complete baseline and re-examination data was missing for an additional 377 individuals, resulting in a study population of 2679 individuals (mean age 72 years, 38% men).

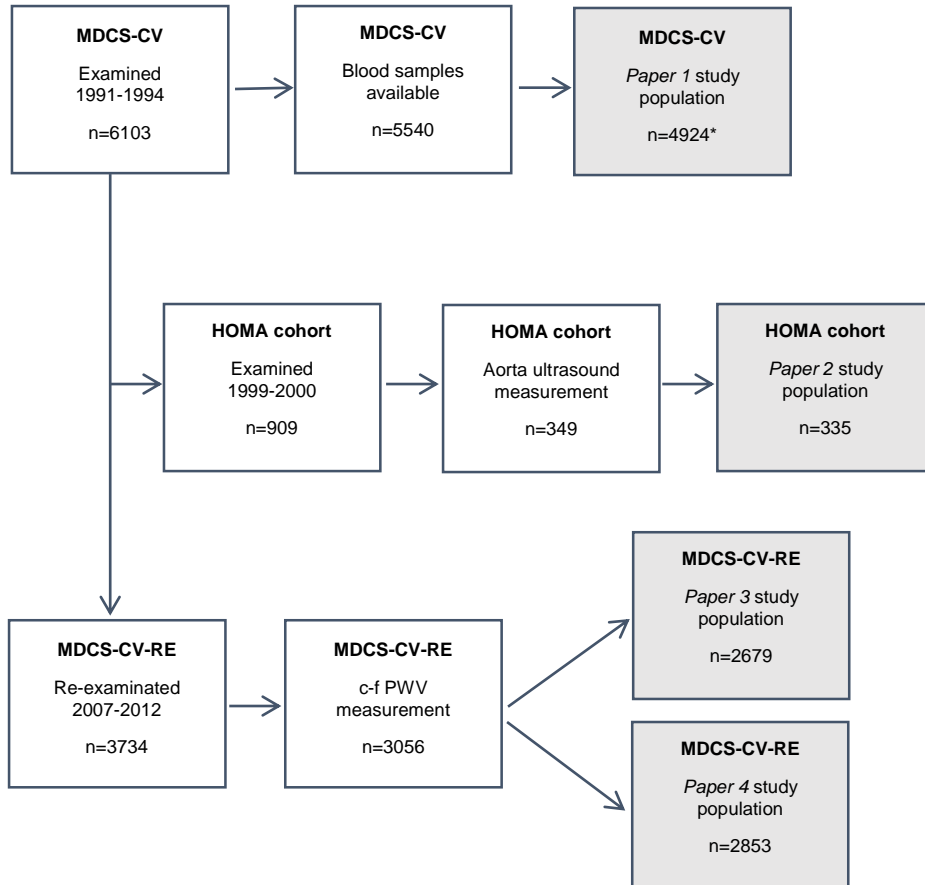


Figure 5. Flow chart of the Cardiovascular Arm of the Malmö Diet and Cancer Study (MDCS-CV), its re-examination (MDCS-CV-RE) and the HOMA-cohort relevant for the paper in this thesis.

* Measurements of cIMT were available in 4888 individuals and measurements of six-graded plaque score were available in 3384 individuals.

Paper 4

Paper 4 is based on MDCS-CV re-examination data with measurements of regional arterial stiffness in 3056 individuals. After excluding individuals with incomplete data, a total of 2853 individuals (mean age 72 years, 40% men) were included in the analyses.

Methods

Clinical measurements

At both MDCS-CV baseline and re-examination, the health examination included a self-administered questionnaire, fasting blood sample and a physical examination.

BMI was calculated as the ratio of weight in kilograms to height in square meters. Waist circumference was measured in centimeters midway between the lowest rib margin and the iliac crest. Information on smoking habits, medical history and pharmacological treatment were retrieved from the questionnaire.

Baseline

BP was measured using a manual sphygmomanometer after 10 minutes of supine rest. Blood samples were collected after an overnight fast. Glucose levels were measured from drawn whole blood. High density lipoprotein cholesterol (HDLc), triglycerides, glucose, HbA_{1c} and insulin were analyzed according to standard procedures. Low density lipoprotein cholesterol (LDLc) was calculated by Friedewald's formula [102]. HOMA-IR was calculated by using the formula: (fasting insulin x fasting glucose)/22.5, where insulin is expressed as mIU/l and glucose as mmol/l [101]. These analyses were performed the same way in the HOMA cohort examinations.

Cystatin C was measured using a particle-enhanced immunonephelometric assay and plasma creatinine was analyzed with the Jaffé method [103]. Estimated glomerular filtration rate (eGFR) was calculated with the combined creatinine–cystatin C described by Inker *et al.* [104].

Re-examination

At the re-examination, BP was measured after five minutes of supine rest with an automatic device (OMRON M5-1 IntelliSense). Glucose levels were measured from a capillary blood sample as plasma glucose with HemoCue (HemoCue AB, Ängelholm, Sweden). The lipid and eGFR analyses were performed the same way

as at the MDCS baseline investigation. An oral glucose tolerance test (OGTT) with repeated plasma glucose measurement 120 minutes after intake of 75g of glucose was performed in nondiabetic individuals (2 h glucose).

Definitions

Hypertension was defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg, or pharmacological BP-lowering drug therapy. Diabetes was defined as fasting blood glucose of at least 6.0 mmol/l (plasma glucose ≥ 7.0 mmol/l) or plasma glucose after OGTT of ≥ 12.2 mmol/l or a history of physician's diagnosis of diabetes mellitus or ongoing pharmacological antidiabetic treatment. Smoking was defined as "current smoking" or "not smoking" in Papers 1 and 2. In Paper 3 smoking status was categorized as "current", "former" and "never".

Paper-specific methods

Paper 1

Analysis of adrenomedullin

In 2008, MR-proADM was analyzed from frozen plasma sampled at the time of the MDCS baseline examination. Analyses were performed using immunoluminometric sandwich assays targeted against amino acids in the midregion of adrenomedullin (BRAHMS AG, Hennigsdorf, Germany) [81]. MR-proADM is produced in equimolar amounts as ADM and its biochemical properties with a longer half-life makes it better suited for analysis [105].

Measurement of atherosclerosis

cIMT and carotid plaques were investigated with ultrasound. Ultrasonographic measurements were performed by highly experienced technicians using B-mode ultrasonography (Acuson XP4; Acuson, Mountain View, California, USA) [106]. The cIMT was measured in the right common carotid artery using a semi-automatic analysis program. Plaque scanning included the three distal centimeters of the right common carotid artery, the bulb and the most proximal centimeter of the internal carotid artery and external carotid artery, respectively. Plaque occurrence and severity was graded according to a six-graded plaque score ranging from zero to five, where zero indicated no plaque and five was the highest plaque score [107]. Intra and inter-observer variability was tested at two separate occasions by three technicians examining IMT in the right common carotid artery in 25 and 41

participants, respectively. Results showed an intra-observer variability of 6-10%, and an inter-observer variability of 8-10%.

Measurement of arteriosclerosis

In Paper 1, brachial PP was used as a measurement of arteriosclerosis. BP was measured with a manual sphygmomanometer after 10 minutes of rest in supine position.

Paper 2

Measurement of local arterial stiffness

In Paper 2, arterial stiffness was measured locally in the abdominal aorta using ultrasound. Ultrasonographic measurements were performed using a phase-locked echo-tracking system (Diamove, Teltec AB, Lund, Sweden) with a spatial resolution of less than 10 μm [108, 109]. The time resolution was 1.15 ms and the smallest detectable movement was 8 μm [108]. The echo-tracking instrument consists of a 3.5 MHz linear array transducer and an ultrasound scanner (EUB 240; Hitachi, Tokyo, Japan) [109]. Two electronic markers automatically identify the posterior and anterior arterial wall, respectively, and follow their pulsatile movements. This procedure was used to assess the maximum and minimum diameters each subject's abdominal aorta, 3–4 cm proximal to the aortic bifurcation. A mean of three readings was recorded. Using a manual sphygmomanometer, brachial BP was measured directly prior to the ultrasound investigation, with the subject in supine position. From the diameter and pressure changes the aortic stiffness index, β , was calculated according to the formula:

$$\text{Stiffness } (\beta) = \ln\left(\frac{SBP}{DBP}\right) \times \frac{Dd}{D_s - Dd}$$

where \ln is the natural logarithm, SBP is systolic blood pressure, DBP is diastolic blood pressure, Dd is the diastolic aortic diameter and D_s is the systolic aortic diameter [67]. Results are based on mean β stiffness index from three measurements.

Paper 3

Measurement of regional arterial stiffness

Regional arterial (aortic) stiffness was measured as c-f PWV on average 261 days after the physical examination and retrieval of blood samples. With the individual in supine position after 5 min of rest, the measurements took place in a quiet

environment. They were performed with SphygmoCor (Atcor Medical, Australia) which is a combined hardware and software using applanation tonometry for pulse registration. The distance was calculated as the suprasternal notch to the umbilicus and from the umbilicus to the measuring point at the femoral artery minus the suprasternal notch to the measuring point at the carotid artery. With simultaneous electrocardiogram (ECG) registration, the software calculates the time from the peak of the R-wave on ECG to the foot of the pulse wave at the carotid and femoral arteries, respectively. The goal was to achieve three measurements (86.7% of cases), although the number of successful measurements per individual varied from one to five. Results are based on mean c-f PWV from these measurements.

The method for distance measurement is a so-called indirect method and no longer recommended according to a consensus document published in 2012 [71] as it was shown to underestimate the real arterial distance calculated by magnetic resonance imaging by 7% [110].

The mean coefficient of variation between c-f PWV measurements was 6.3% (\pm SD 4.4). Inter-observer variability has been tested twice, both times by two technicians. At one occasion, c-f PWV was measured in 17 participants showing 5.0% (\pm SD 4.0) difference between observers. At a second occasion, an examination of 13 individuals resulted in a 7.2% (\pm SD 9.9) difference between measurements of the two observers.

Paper 4

Measurement of regional arterial stiffness

The same measurements of regional arterial stiffness with c-f PWV previously described for Paper 3 were also used in Paper 4.

Genotyping

Blood samples collected at MDCS baseline were used for genotyping. The SNPs were genotyped using a MALDI-TOF mass spectrometer (Sequenom Mass Array, Sequenom, San Diego, USA). SNPs that failed this analysis were analyzed individually using the Taqman or KASPar allelic discrimination method on an ABI 790HT (applied Biosystems, Life Technologies, Carlsbad, CA, USA). SNAP version 2.2 was used to find proxy SNPs in cases where matching SNPs were not found. Imputation was used in cases of SNP genotype failure, and individuals with less than 60% successful genotyped SNPs were excluded. SNPs with a genotype success rate of less than 90% or deviation from the Bonferroni-corrected Hardy-Weinberg Equilibrium in each set of SNPs for each trait were excluded. At least 25% of individuals were also genotyped with a different method, Human

OmniExpress Bead Chip (Illumina, San Diego, CA, USA), to check for concordance, which was more than 98% for all included SNPs.

Construction of genetic risk scores

Construction of GRSs for SBP (29 SNPs), BMI (31 SNPs), LDLc (32 SNPs), HDLc (41 SNPs), triglycerides (26 SNPs), fasting plasma glucose (FPG) (15 SNPs) and type 2 diabetes (T2D) (48 SNPs) were performed using publications from large multicenter GWASs [111-119]. The SNPs for FPG were all discovered in non-diabetic individuals [116]. Of the 15 SNPs included in the FPG GRS, seven overlapped with the SNPs in the T2D GRS. In addition, a modified GRS for T2D without the seven SNPs overlapping in the FPG GRS was created. This GRS is referred to as T2D41 GRS (as it includes 41 SNPs). Also for the other GRSs, a few SNPs had shown GWAS significance for several traits and, thus, were included in several scores. The genotype at each locus was coded as 0, 1 or 2 depending on the number of alleles previously shown to increase the risk factor in question. With information from previous GWAS publications, each allele was weighted according to the estimated effect size.

Statistical methods

Statistical calculations were performed using IBM SPSS Statistics, version 19-23 (IBM Corp., Armonk, New York, USA). In Paper 4, PLINK (version 1.07) and R (version 3.31) were also used. Correlations were analyzed in crude models using Spearman's rank correlation test. In adjusted models, multiple linear regression analyses were performed. For multiple linear regression analyses, skewed variables including c-f PWV, β stiffness index, triglycerides, HOMA-IR, HbA_{1c}, FPG and 2 h glucose were logarithm transformed to achieve normal distributions. In Paper 2, HDLc was also logarithm transformed and in Paper 4 both HDLc and LDLc were logarithm transformed. In adjusted analyses including c-f PWV, adjustments for MAP was always performed as MAP influences the intrinsic elastic properties of the arterial wall [22, 23]. In Paper 3, multiple linear regression analyses with c-f PWV also included adjustment for heart rate (HR). Mann-Whitney U-tests were used to test for differences between two groups. ANOVA was used in Paper 3 to test for differences between more than two groups and in the event of significant differences, was further analyzed with Tukey's post-hoc analysis. For categorical variables, chi-square test was used. In adjusted models ANCOVA was used for continuous variables and binary logistic regression was used for categorical variables. A p-value <0.05 was considered significant, except for in Paper 3 where the p-value was sharpened to <0.01 due to a larger number of variables tested for association.

Statistical analysis for Mendelian randomization

Multiple linear regression was used to calculate the associations between each GRS and its respective trait, as well as the associations between each trait and c-f PWV.

Two different methods were used in the subsequent analyses.

First, the associations between c-f PWV and each GRS were calculated. This was done using multiple linear regression adjusting for age, sex and MAP. Binary logistic regression was used to calculate the odds ratio of T2D GRS for T2D. When the IV was SBP GRS, ongoing BP-lowering drug therapy was added to the analysis and additionally, sensitivity analysis without BP-lowering drug therapy was performed. When the IV was LDLc GRS, HDLc GRS or triglyceride GRS, lipid-lowering treatment was added to the analysis and additionally, sensitivity analysis without individuals on lipid-lowering treatment was performed. When the IV was FPG GRS, individuals with diabetes were excluded in sensitivity analysis.

Secondly, an inverse-variance weighted MR regression was performed [118, 119]. This was done as a complementary approach in order to correct for potential bias of pleiotropic effects of an SNP on any of the other studied traits without removing any of the overlapping SNPs. In this approach, the β -coefficients from the multiple linear regression of each of the 183 SNPs on c-f PWV were regressed on the β -coefficients from the multiple linear regression of the same SNPs on each trait. This regression was inverse-variance weighted using standard errors of each SNP-PWV association and the intercept was fixed to zero.

Results

Paper 1

The study population consisted of 4924 individuals. Of those, measurements of cIMT and a six-graded plaque score were available in 4888 and 3384 individuals, respectively. The characteristics of the study population are presented in Table 2.

Table 2. Characteristics of the study population in Paper 1 (n=4924).

Characteristics	Mean (\pm SD)
Age (years)	58 (6)
SBP (mmHg)	141 (19)
DBP (mmHg)	87 (9)
PP (mmHg)	54 (14)
BMI (kg/m ²)	25.7 (3.9)
LDLc (mmol/L)	4.17 (0.98)
MR-proADM (mmol/L)	0.46 (0.13)
cIMT (mm) (n=4888)	0.74 (0.16)
Mean plaque score (n=3384)	1.7 (1.7)
Men, n (%)	1991 (40.4)
Current smoking, n (%)	1311 (26.6)
Diabetes mellitus, n (%)	377 (7.7)
Hypertension, n (%)	3196 (64.9)

SBP, systolic blood pressure; DBP, diastolic blood pressure; PP, pulse pressure; MR-proADM, mid-regional pro-adrenomedullin; LDLc, low density lipoprotein cholesterol; cIMT, intima-media thickness in right common carotid artery; BMI, body mass index.

Mean levels of MR-proADM were lower among normotensive individuals than among hypertensive individuals (0.43 vs. 0.48 mmol/l, $p < 0.001$).

Univariate analysis showed a positive, statistically significant relationship between levels of MR-proADM and PP, cIMT and carotid plaques, respectively. These

differences remained after adjustment for age, sex, BMI, hypertension, diabetes, LDLc, smoking and eGFR. The associations between MR-proADM and both PP and cIMT existed for both sexes, whereas the association with plaque score was only significant among women. For hypertensive individuals, there was an association (*Model 2*) between MR-proADM and PP ($\beta=0.07$, $p=0.001$), cIMT ($\beta=0.05$, $p=0.014$) and plaque score ($\beta=0.07$, $p=0.006$). In the normotensive group, the association (*Model 2*) was statistically significant for PP ($\beta=0.13$, $p<0.001$) but not for cIMT ($p=0.29$) or plaque score ($p=0.35$).

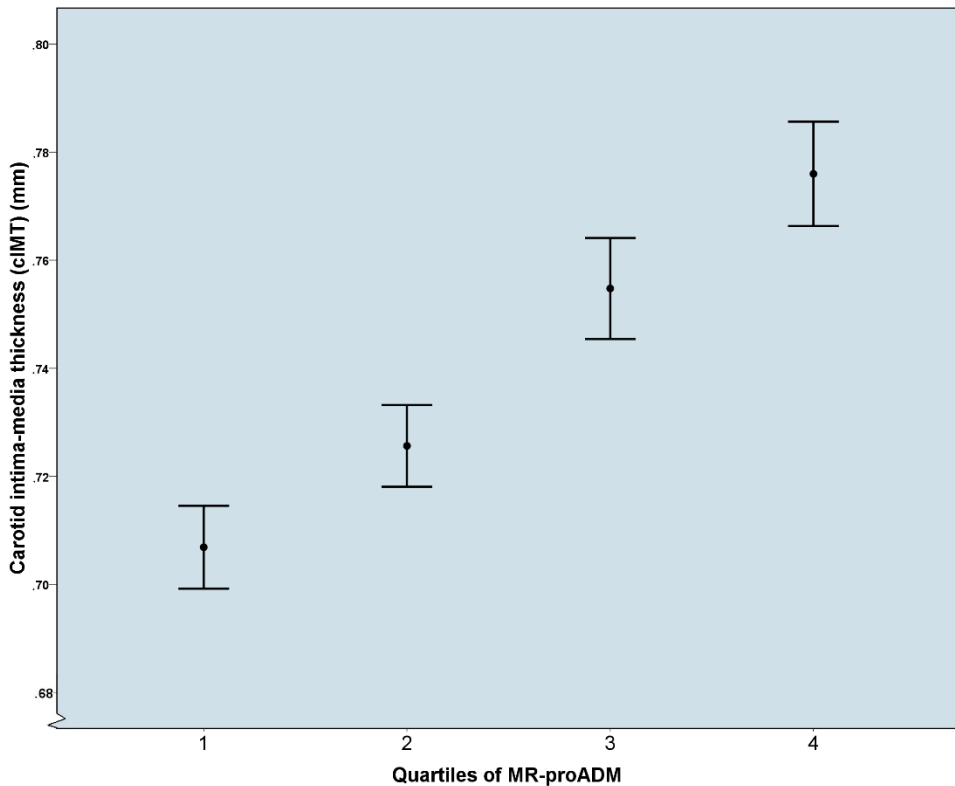


Figure 6. Mean and 95% confidence interval of carotid intima–media thickness in different quartiles of mid-regional pro-adrenomedullin. MR-proADM, mid-regional pro-adrenomedullin.

Table 3. Association between mid-regional part of pro-adrenomedullin and pulse pressure, carotid intima-media thickness and carotid plaques respectively.

	Univariate model		Adjusted model 1		Adjusted model 2	
	r	p	β	p	β	p
Pulse pressure	0.21	<0.001	0.06	<0.001	0.07	<0.001
cIMT	0.16	<0.001	0.04	0.004	0.04	0.007
Carotid plaques	0.15	<0.001	0.05	0.004	0.06	0.003

Adjusted *Model 1*: Adjusted for age, sex, body mass index, hypertension, diabetes, smoking and low density lipoprotein cholesterol.

Adjusted *Model 2*: Adjusted *Model 1* + adjustment for estimated glomerular filtration rate. cIMT, carotid intima-media thickness

In additional analysis, not originally presented in Paper 1, the correlation between MR-proADM and c-f PWV, the latter measured at MDCS re-examination, was analyzed. The study population included 2654 individuals and mean follow-up time was 17 years. In a model including adjustment for MAP and HR at re-examination, MR-proADM was significantly associated with c-f PWV ($\beta=0.17$, $p<0.001$). This significance was lost ($\beta=0.03$, $p<0.14$) after further adjustment for age, sex, BMI, hypertension, diabetes, smoking, LDLc, and eGFR at MDCS-CV baseline.

Paper 2

The mean follow-up time from MDCS-CV baseline to the HOMA-cohort investigation was 6.7 years (\pm SD 0.7). The characteristics of the study population are presented in Table 4. Of the 335 individuals with complete data, five individuals were excluded from further analysis, including two individuals with β stiffness index more than 60 (extreme outliers) and three individuals with PP at follow-up of less than 20 mmHg (outliers and suspected incorrect measurements). The β stiffness index values showed a positively skewed distribution and were significantly higher in men than in women (median 14.6 vs 10.8, $p<0.001$).

Table 4. Characteristics of the study population in Paper 2 at baseline and follow-up, stratified by gender (n=335).

Characteristics	Baseline		Follow-up	
	Men (n=139)	Women (n=196)	Men (n=139)	Women (n=196)
Age (years)	58 (6)	58 (6)	64 (6)	65 (6)
SBP (mmHg)	143 (19)	140 (18)	139 (20)	138 (19)
DBP (mmHg)	88 (9)	86 (9)	84 (8)	81 (8)
BMI (kg/m ²)	26.1 (3.5)	26.6 (4.0)	26.6 (3.5)	27.2 (4.2)
Waist circumference (cm)	93 (10)	80 (10)	96 (10)	87 (11)
β stiffness index	n.a.	n.a.	14.6 [10.7-20.8]	10.8 [8.0-15.7]
LDLc (mmol/L)	4.14 (0.86)	4.29 (1.04)	3.71 (0.73)	3.76 (0.91)
HDLc (mmol/L)	1.23 (0.32)	1.48 (0.38)	1.38 (0.34)	1.63 (0.40)
Triglycerides (mmol/L)	1.26 [0.93-1.82]	1.22 [0.88-1.59]	1.15 [0.80-1.67]	1.13 [0.83-1.60]
Fasting glucose (mmol/L)	5.0 [4.7-5.4]	4.9 [4.6-5.3]	5.2 [4.9-5.8]	5.1 [4.8-5.6]
HOMA-IR	1.88 [1.25-2.61]	1.75 [1.09-2.66]	2.16 [1.29-3.30]	1.83 [1.29-3.06]
Current smoking, n (%)	40 (29)	40 (20)	41 (30)	39 (20)
Blood pressure-lowering drug therapy, n (%)	21 (15)	32 (16)	28 (20)	55 (28)

For continuous variables, normally distributed characteristics expressed with mean (\pm SD) and others expressed as median [first quartile-third quartile].

SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; n.a., not available.

In unadjusted models, using Spearman's univariate correlation, there were statistically significant, positive correlations between β stiffness index and both baseline and follow-up waist circumference (baseline: $r=0.35$, $p<0.001$, follow-up: 0.32 , $p<0.001$) as well as triglycerides (baseline: $r=0.15$, $p=0.005$, follow-up: $r=0.15$, $p=0.006$). HDLc at baseline and follow-up was inversely associated with aortic stiffness (baseline: $r= -0.28$, $p<0.001$, follow-up: $r= -0.26$, $p<0.001$). HOMA-IR was positively associated with aortic stiffness according to both baseline and follow-up measurements (baseline: $r=0.19$, $p=0.001$, follow-up: $r=0.16$, $p=0.004$), but for HOMA-IR none of these results were statistically significant in the male subgroup. There were no associations between LDLc and β stiffness index in either sex.

Results from multiple linear regression analysis are presented in Table 5. The β stiffness index was statistically significant and positively associated with age and sex. In all subjects and in the female subgroup, HDLc at both baseline and follow-up was negatively associated with β stiffness index. The β stiffness index in the female subgroup was positively associated with HOMA-IR at baseline, but not at

follow-up. In the male subgroup, β stiffness index was positively associated with waist circumference but not with HOMA-IR or HDLc.

Table 5. Multiple linear regression analysis for baseline and follow-up determinants of the dependent variable, aortic stiffness (β stiffness index), for all individuals and stratified by gender.

		All		Men		Women	
		β	p	β	p	β	p
Follow-up	Age at follow-up	0.34	<0.001	0.27	0.002	0.42	<0.001
	Female sex	-0.18	0.001				
	Waist circumference	0.14	0.03	0.21	0.04	0.09	0.24
	HOMA-IR	-0.03	0.68	-0.05	0.64	-0.01	0.88
	Triglycerides	-0.02	0.80	0.01	0.90	-0.05	0.52
	HDLc	-0.19	0.002	-0.12	0.23	-0.22	0.004
	Smoking	-0.01	0.84	0.03	0.74	-0.04	0.52
Baseline	Age at baseline	0.32	<0.001	0.23	0.01	0.42	<0.001
	Female sex	-0.18	0.005				
	Waist circumference	0.09	0.23	0.23	0.04	0.03	0.72
	HOMA-IR	0.06	0.33	-0.14	0.21	0.18	0.03
	Triglycerides	-0.06	0.36	0.04	0.67	-0.16	0.07
	HDLc	-0.16	0.01	-0.04	0.73	-0.23	0.005
	Smoking	-0.01	0.81	0.04	0.68	-0.05	0.47

All variables from baseline or follow-up fitted in one multiple regression model with further adjustment for ongoing blood pressure-lowering drug treatment at the ultrasound examination.

HOMA-IR, homeostatic model assessment of insulin resistance; HDLc, high density lipoprotein cholesterol.

Paper 3

Mean follow-up time from MDCS-CV baseline examination to c-f PWV measurement at MDCS-CV re-examination was 16.9 years. Median c-f PWV was 10.1 m/s, significantly higher in men than in women (10.4 vs. 9.9 m/s, $p < 0.001$). In all, 25 individuals (0.9% of the study population) had an eGFR below 30 ml/min/1.73m² at re-examination. The characteristics of the study population are presented in Table 6.

Table 6. Characteristics of the study population in Paper 3 at baseline and follow-up, stratified by gender (n=2679).

Characteristics	Baseline		Follow-up	
	Men (n=1007)	Women (n=1672)	Men (n=1007)	Women (n=1672)
Age (years)	56.0 (5.8)	56.0 (5.6)	72.0 (5.7)	72.2 (5.5)
SBP (mmHg)	140 (17)	137 (18)	136 (17)	135 (18)
DBP (mmHg)	88 (9)	85 (9)	76 (9)	75 (9)
BMI (kg/m ²)	25.7 (3.1)	25.0 (3.7)	26.8 (3.7)	26.5 (4.4)
Waist circumference (cm)	91 (9)	76 (9)	98 (10)	87 (11)
Fasting glucose (mmol/l)	5.0* [4.7-5.3]	4.8* [4.5-5.1]	6.0† [5.5-6.6]	5.7† [5.3-6.2]
HbA1c (%)	4.7 [4.5-5.0]	4.8 [4.5-5.0]	n.a.	n.a.
HOMA-IR (n=2636)	1.46 [0.98-2.12]	1.23 [0.82-1.74]	n.a.	n.a.
Triglycerides (mmol/l)	1.23 [0.93-1.69]	1.02 [0.78-1.38]	1.00 [0.70-1.30]	0.90 [0.70-1.30]
LDLc (mmol/l)	4.12 (0.87)	4.12 (1.02)	3.14 (0.88)	3.46 (0.93)
HDLc (mmol/l)	1.23 (0.29)	1.53 (0.36)	1.25 (0.36)	1.56 (0.43)
eGFR (ml/min/1.73m ²) (n=2418)	95 (13)	88 (12)	70 (15)	66 (14)
c-f PWV (m/s)	n.a.	n.a.	10.4 [9.0-12.1]	9.9 [8.7-11.5]
Diabetes n (%)	72 (7)	63 (4)	165 (16)	189 (11)
Current smoking, n (%)	229 (23)	369 (22)	102 (10)	146 (9)
Former smoking, n (%)	439 (44)	474 (28)	542 (54)	653 (39)
Lipid-lowering drug therapy, n (%)	26 (3)	28 (2)	336 (33)	437 (26)
Blood pressure-lowering drug therapy, n (%)	140 (14)	193 (12)	571 (57)	885 (53)

* Blood glucose

† Plasma glucose

For continuous variables, normally distributed characteristics expressed with mean (\pm SD) and others expressed as median [first quartile-third quartile]. SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; c-f PWV, carotid-femoral pulse wave velocity; n.a., not available.

Baseline predictors of arterial stiffness

The baseline predictors of c-f PWV are presented in Table 7. When adjusting for cardiovascular risk factors (*Model 2*), c-f PWV was significantly and positively associated with the following baseline variables: age, male sex, BMI, waist circumference, SBP, PP, fasting glucose, HbA_{1c} and triglycerides, but negatively associated with HDLc. Except for BMI, which was not significant for men, all associations were significant for both sexes. Figure 7 displays c-f PWV in different

BMI categories at baseline. There was no prediction of c-f PWV from baseline eGFR, LDLc levels or smoking status after adjustment in *Model 2*.

Table 7. Multiple linear regression analysis for baseline determinants of the dependent variable, carotid–femoral pulse wave velocity.

Determinant	<i>Model 1</i>		<i>Model 2</i>					
	Both sexes		Both sexes		Men		Women	
	β	p	β	p	β	p	β	p
Age	0.40	<0.001	0.38	<0.001	0.42	<0.001	0.36	<0.001
Male sex	0.11	<0.001	0.10	<0.001				
SBP	0.15	<0.001	0.11	<0.001	0.13	<0.001	0.11	<0.001
DBP	0.08	<0.001	0.02	n.s.	0.05	n.s.	0.01	n.s.
PP	0.15	<0.001	0.13	<0.001	0.13	<0.001	0.13	<0.001
BMI	0.10	<0.001	0.08	<0.001	0.07	0.011	0.10	<0.001
Waist circumference	0.18	<0.001	0.17	<0.001	0.12	<0.001	0.14	<0.001
Fasting glucose	0.17	<0.001	0.13	<0.001	0.14	<0.001	0.12	<0.001
HbA _{1c}	0.07	<0.001	0.06	<0.001	0.08	0.002	0.04	0.048
HOMA-IR	0.14	<0.001	0.10	<0.001	0.10	0.001	0.10	<0.001
Triglycerides	0.14	<0.001	0.10	<0.001	0.11	<0.001	0.09	<0.001
LDLc	0.04	n.s.	0.03	n.s.	0.04	n.s.	0.03	n.s.
HDLc	-0.14	<0.001	-0.08	<0.001	-0.07	0.005	-0.08	<0.001
eGFR	0.05	0.006	0.03	n.s.	0.05	n.s.	0.02	n.s.
Current smoking	0.01	n.s.	0.02	n.s.	0.02	n.s.	0.01	n.s.
Former smoking	0.04	0.02	0.02	n.s.	0.03	n.s.	0.01	n.s.

Model 1: The model includes age, MAP, and HR. The rest of the determinants were individually entered into the model together with covariates included in *Model 1*.

Model 2: The model includes age, sex, BMI, current smoking, ongoing blood pressure-lowering drug therapy, ongoing lipid-lowering drug therapy, HR, and MAP. Fasting glucose, HbA_{1c}, HOMA-IR, triglycerides, HDLc, LDLc, and eGFR were individually entered into the model together with covariates included in *Model 2*. When waist circumference was entered in *Model 2*, BMI was excluded from the model.

SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; HOMA-IR, homeostatic model assessment of insulin resistance; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; n.s., not significant; MAP, mean arterial pressure; HR, heart rate.

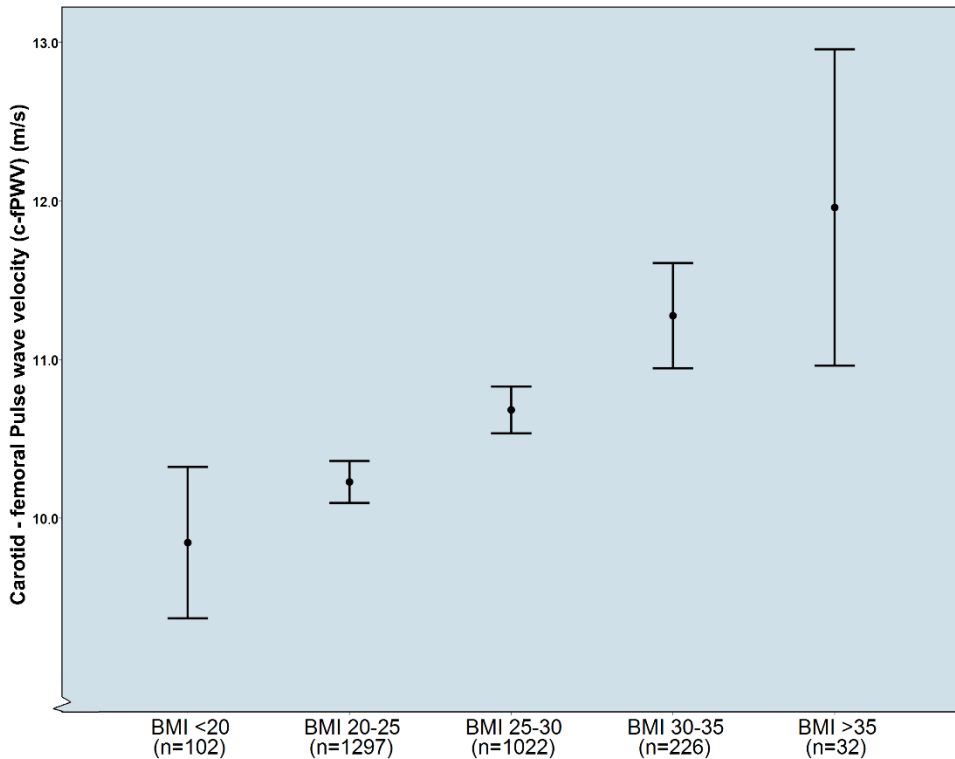


Figure 7. Mean and 95% confidence interval of carotid-femoral pulse wave velocity stratified by body mass index (kg/m^2) at the baseline of Malmö Diet and Cancer study Cardiovascular arm. BMI, body mass index.

Cross-sectional relationship with arterial stiffness

The results from the cross-sectional association analyses between cardiovascular risk markers and c-f PWV are presented in Table 8. The same variables that were predictive of c-f PWV were also associated with c-f PWV in the cross-sectional analyses with two exceptions: In *Model 1*, a negative association was found for LDLc and no association was found for eGFR in *Model 1* or 2. In sensitivity analysis, when excluding individuals with ongoing lipid-lowering drug therapy at follow-up, there were no significant associations between c-f PWV and LDLc. The relationship between FPG and c-f PWV was also present in FPG levels below the threshold for diabetes, which is illustrated in Figure 8. The variable 2h glucose, available only in individuals without diagnosed diabetes, was associated with c-f PWV ($\beta=0.13$, $p<0.001$).

Table 8. Multiple linear regression analysis for cross-sectional associations with the dependent variable, carotid–femoral pulse wave velocity.

Determinant	Model 1		Model 2					
	Both sexes		Both sexes		Men		Women	
	β	p	β	p	β	p	β	p
Age	0.40	<0.001	0.39	<0.001	0.43	<0.001	0.37	<0.001
Male sex	0.12	<0.001	0.11	<0.001				
BMI	0.11	<0.001	0.08	<0.001	0.07	0.008	0.10	<0.001
Waist circumference	0.18	<0.001	0.14	<0.001	0.11	<0.001	0.13	<0.001
FPG	0.15	<0.001	0.09	<0.001	0.10	<0.001	0.09	<0.001
Triglycerides	0.13	<0.001	0.10	<0.001	0.09	0.001	0.11	<0.001
LDLc	-0.10	<0.001	-0.02	n.s.	-0.04	n.s.	0.00	n.s.
HDLc	-0.15	<0.001	-0.08	<0.001	-0.08	0.003	-0.08	0.001
eGFR	-0.02	n.s.	0.00	n.s.	-0.01	n.s.	0.01	n.s.
Current smoking	-0.01	n.s.	0.00	n.s.	-0.01	n.s.	0.00	n.s.
Former smoking	0.00	0.017	0.01	n.s.	0.03	n.s.	-0.01	n.s.
MAP	0.31	<0.001	0.29	<0.001	0.31	<0.001	0.28	<0.001
HR	0.18	<0.001	0.20	<0.001	0.24	<0.001	0.18	<0.001

Model 1: The model includes age, MAP, and HR. The rest of the determinants were individually entered into the model together with covariates included in *Model 1*.

Model 2: The model includes age, sex, BMI, current smoking, ongoing blood pressure-lowering drug therapy, ongoing lipid-lowering drug therapy, HR, and MAP. Fasting glucose, triglycerides, HDLc, LDLc, and eGFR were individually entered into the model together with covariates included in *Model 2*. When waist circumference was entered in *Model 2*, BMI was excluded from the model.

SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; FPG, fasting plasma glucose; LDLc, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; MAP, mean arterial pressure; HR, heart rate; n.s., not significant.

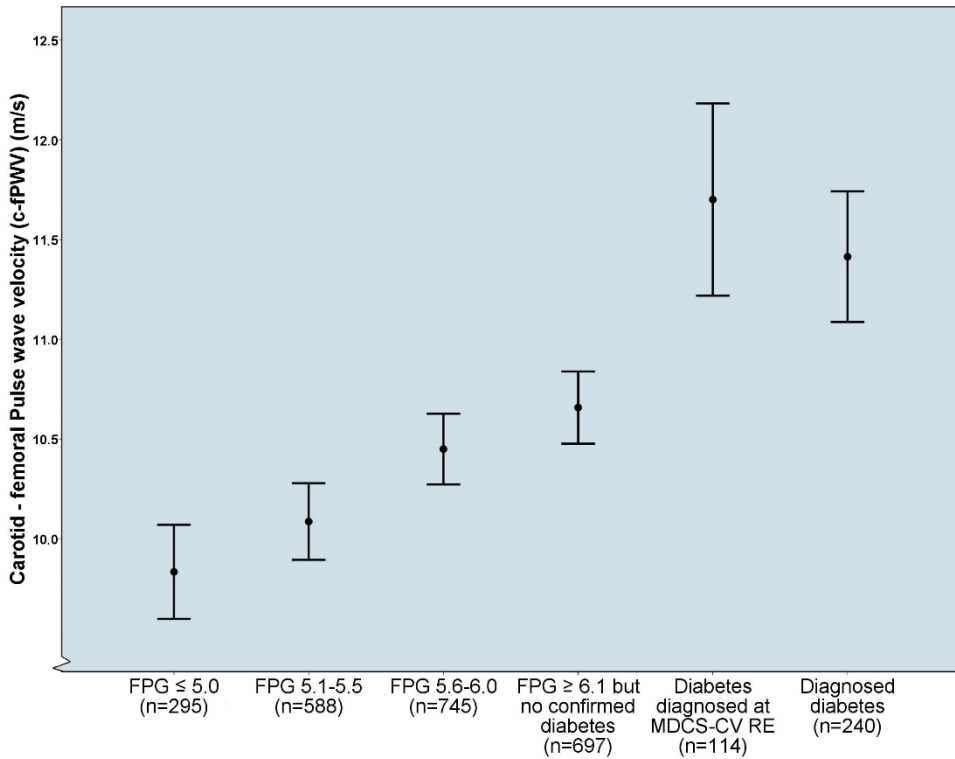


Figure 8. Mean and 95% confidence interval of carotid-femoral pulse wave velocity stratified for fasting plasma glucose (mmol/L) or diabetes status at the MDCS-SV RE. FPG, fasting plasma glucose; MDCS-SV RE, re-examination of Malmö Diet and Cancer study Cardiovascular arm.

Paper 4

The study population, presented in Table 9, consisted of 2853 individuals from the MDCS-CV RE. All traits were significantly associated with c-f PWV. Each GRS, except for SBP GRS, was significantly associated with its respective trait.

There were statistically significant associations between c-f PWV and both FPG GRS and T2D GRS but not between T2D41 GRS and c-f PWV. In sensitivity analyses excluding individuals with diabetes, the significance for FPG GRS remained. There were borderline significant associations for SBP GRS ($\beta = -0.03$, $p = 0.05$) and triglyceride GRS ($\beta = 0.03$, $p = 0.05$), but none of these remained in sensitivity analyses excluding individuals with BP-lowering treatment and lipid-lowering treatment, respectively. For the other GRSs there were no statistically significant associations with c-f PWV, as presented in Table 10.

Table 9. Characteristics of the study population in Paper 4 at baseline and follow-up (n=2853).

Characteristics	Mean (\pm SD)
Age (years)	72.1 (5.5)
SBP (mmHg)	136 (17)
DBP (mmHg)	76 (9)
BMI (kg/m ²)	26.7 (4.2)
LDLc (mmol/l)	3.3 (0.9)
HDLc (mmol/l)	1.4 (0.4)
Triglycerides (mmol/l)	1.0 [0.7-1.3]
FPG (mmol/l)	5.8 [5.4-6.4]
c-f PWV (m/s)	10.1 [8.8-11.8]
Men, n (%)	1147 (40.2)
Diabetes, n (%)	401 (14.1)
Blood pressure-lowering drug therapy, n (%)	1539 (53.9)
Lipid-lowering drug therapy, n (%)	826 (29.0)

For continuous variables, normally distributed characteristics expressed with mean (\pm SD) and others expressed as median [first quartile-third quartile].

SBP, systolic blood pressure; DBP, diastolic blood pressure; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; FPG, fasting plasma glucose; c-f PWV, carotid-femoral pulse wave velocity.

Table 10. Association between genetic risk scores and carotid-femoral pulse wave velocity adjusted for age, sex, mean arterial pressure and ongoing blood pressure-lowering treatment.

GRS	β PWV*	p*	β PWV†	p†
BMI	0.01	0.70	n.a.	n.a.
SBP	-0.03	0.05	-0.04	0.10
LDLc	-0.004	0.78	0.02	0.42
HDLc	-0.02	0.13	-0.02	0.24
Triglycerides	0.03	0.05	0.01	0.56
FPG	0.03	0.03	0.04	0.02
T2D	0.03	0.04	n.a.	n.a.
T2D41	0.03	0.07	n.a.	n.a.

* β for LDLc, HDLc and triglycerides also adjusted for lipid-lowering treatment.

† Sensitivity analyses excluding individuals on lipid-lowering treatment in the lipid analyses, blood pressure-lowering treatment in the blood pressure analyses, and diabetes in the FPG analysis.

GRS, genetic risk score; SBP, systolic blood pressure; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; FPG, fasting plasma glucose; T2D, type 2 diabetes; T2D41, T2D GRS without seven single nucleotide polymorphisms overlapping in the FPG GRS; n.a., not applicable..

A total of 183 unique SNPs ($r^2 < 0.2$) that were reported in GWAS to associate with SBP, BMI, LDLc, HDLc, triglycerides, FPG and T2D, were included in the inverse-

variance weighted MR. There was a significant association between c-f PWV β coefficients and FPG β coefficients ($p=0.006$), but not with T2D β coefficients ($p=0.88$). There were no significant associations between c-f PWV β coefficients and BMI, SBP, LDLc, HDLc or triglycerides β coefficients. Table 11 illustrates this. When excluding eight SNPs associated with FPG but not with T2D, FPG β coefficients were still significantly associated with PWV β coefficients ($p=0.018$) but T2D results remained insignificant ($p=0.33$).

Table 11. Inverse-variance weighted Mendelian randomization of cardiometabolic traits and carotid-femoral pulse wave velocity adjusted for age, sex, mean arterial pressure and blood pressure-lowering treatment at follow-up.

	β_{PWV^1}		β_{PWV^2}	
	Direction	p	Direction	p
β_{BMI}	-	0.82	-	0.80
β_{SBP}	+	0.98	+	0.91
β_{LDLc}	-	0.32	-	0.27
β_{HDLc}	-	0.73	-	0.75
β_{TG}	+	0.18	+	0.16
β_{FPG}	+	0.006	+	0.006
β_{T2D}	+	0.88	+	0.88

1. β_{SBP} obtained after adjustment for age, sex and antihypertensive treatment at baseline. β_{LDLc} β_{HDLc} β_{TG} obtained after adjustment for age, sex and lipid-lowering treatment at baseline.

2. Sensitivity analyses excluding individuals on lipid-lowering medication in the lipid analyses and antihypertensive medication in the blood pressure analyses.

PWV, pulse wave velocity; SBP, systolic blood pressure; LDLc, low density lipoprotein cholesterol; HDLc, high density lipoprotein cholesterol; TG, triglycerides, FPG, fasting plasma glucose; T2D, type 2 diabetes.

Discussion

Markers of vascular ageing

Arterial stiffness describes the stiffening of the arterial wall, a well-documented risk factor of cardiovascular events and mortality. It is known to increase with age and is inter-correlated with BP both for mechanistic and pathophysiological reasons. The results from **Paper 3** show that obesity, impaired glucose metabolism, triglycerides and HDLc were all predictors of regional arterial stiffness after a follow-up period of 17 years. These markers also had cross-sectional relationships with arterial stiffness. No associations were found between arterial stiffness and smoking or LDLc. The results are partially concordant with those from **Paper 2** where local abdominal aortic stiffness was measured in a smaller study population. The results from **Paper 2** are primarily discussed in a specific section.

Obesity

In **Paper 3**, BMI and waist circumference, both at baseline and follow-up, were associated with arterial stiffness. The results were present already in BMI categories below obesity (BMI <30 kg/m²) where overweight individuals (BMI 25-30 kg/m²) had higher c-f PWV at follow-up than individuals with a normal BMI (BMI 20-25 kg/m²). Obese individuals are known from several previous studies to have relatively increased arterial stiffness, a pattern that emerges already in adolescence [120, 121]. There was a tendency of waist circumference to be more strongly correlated to arterial stiffness than BMI. Similar findings have been previously published [122] pointing towards abdominal obesity as the underlying factor behind the association.

A meta-analysis showing a reduced arterial stiffness after diet or exercise-induced weight loss (8% of initial body weight) supports the hypothesis that obesity is causally linked to arterial stiffness [123]. This was accompanied by a reduction in BP, although the study design did not allow any conclusion regarding the direction of this correlation. Obesity in mice, induced by a high-fat, high-sucrose diet, has been shown to result in an increased PWV, preceding the development of hypertension [124]. The aortas from the obese mice had increased levels of pro-

inflammatory cytokines such as tumor necrosis factor- α (TNF- α), reduced endothelial NO-production and increased levels of tissue transglutaminase-2 (TG-2) [124]. TG-2, found in vascular cells, catalyzes a reaction forming strong N- ϵ -(γ -glutamyl)-lysine bonds between extracellular matrix proteins, thereby increasing stiffness. The perivascular adipose tissue (PVAT) has been suggested to play a role in the pathogenesis through paracrine effects on the arterial wall, thus promoting inflammation [125].

Overall, results from **Paper 3** together with findings from several previous publications, indicate that obesity is associated with and presumably involved in the pathogenesis of arterial stiffness.

Lipids

In **Paper 3**, arterial stiffness was positively associated to triglycerides and negatively associated to HDLc (dyslipidemia) at both baseline and follow-up. As discussed in the previous section, obesity is linked to arterial stiffness but the results for HDLc and triglycerides remained after full adjustment including BMI.

In a systematic review from 2009, triglycerides were associated with arterial stiffness in only one out of 38 studies [55]. In the same review, HDLc was associated with decreased arterial stiffness in only four of 37 studies. However, many of these studies were small or carried out in specific populations. In a cross-sectional study from the MARE consortium, including 20,570 individuals from nine cohorts, levels of PWV were compared between several clusters of the metabolic syndrome (high triglycerides, FPG, BP and waist circumference and low HDLc) [61]. The study concluded that 3 of the metabolic syndrome clusters, two of which involved high triglycerides, were associated with increased arterial stiffness. Furthermore, the findings from **Paper 3** are similar to findings in 3679 individuals from the British occupation-based cohort Whitehall II [126]. Published results from this study have shown both HDLc and triglycerides were predictors of c-f PWV after a follow-up time of 16 years.

Abdominal obesity and insulin resistance both result in increased levels of free fatty acids, leading to the formation and release of very low density lipoprotein (VLDL) particles rich in triglycerides and decreased levels of HDLc [127]. Since obesity, insulin resistance and dyslipidemia are closely linked, it is difficult to conclude if triglycerides or HDLc are actually involved in the pathogenesis, or if they only have a status as markers. HDLc affects and is affected by inflammation [128, 129], and thus its association with arterial stiffness could be mediated through chronic inflammation. Genetic analysis and RCTs suggest no causal link between HDLc and CVD making a direct involvement of HDLc in arterial stiffening pathogenesis less

likely [130]. For triglycerides on the other hand, recent evidence suggests a causal role in atherosclerosis, inflammation and all-cause mortality [131].

In **Paper 3**, baseline LDLc did not predict arterial stiffness. In cross-sectional analyses, LDLc was negatively associated with arterial stiffness but this relationship disappeared after adjustment and in sensitivity analyses excluding individuals with ongoing lipid-lowering drug therapy. As the population receiving lipid-lowering drugs (29% at follow-up) are more likely to have CVD they also have higher c-f PWV. This explains the negative association between follow-up LDLc levels and c-f PWV before full adjustment. In the previously mentioned review from 2009, none of the 18 studies investigating relationship between arterial stiffness and LDLc showed a positive relationship [55].

The absence of an association between LDLc and arterial stiffness found both in **Paper 2** and **Paper 3** suggests that LDLc does not play an independent role in the development of arteriosclerosis. This is in strong contrast to the well documented role of LDLc in atherosclerosis pathogenesis [132].

Smoking

Tobacco smoking is known to be an important risk factor for atherosclerosis [133], but the potential role of smoking in the pathophysiology of arterial stiffness is more dubious. Some previous studies report an association between the effect of chronic smoking and arterial stiffness [134-136], but many studies do not [126, 136]. Since nicotine exposure increases HR, adjustment for HR and MAP at the time of the PWV measurement is essential for any analysis investigating the effect of chronic smoking. One study using the β -stiffness index showed an increased abdominal aortic stiffness in female chronic smokers compared to non-smokers whereas there was no such difference among men [137]. Results from **Paper 2** and **Paper 3** show no associations between current or former smoking status and arterial stiffness.

Glomerular filtration rate

Results from **Paper 3** show that eGFR at baseline was predictive of arterial stiffness. However, after full adjustment this association was lost and there were no associations remaining between eGFR and arterial stiffness at baseline or follow-up. In individuals with CKD stages 2-4, results regarding the relationship between arterial stiffness and GFR have been conflicting [64]. In two of the studies still demonstrating such a relationship, eGFR could only explain a small fraction, around 2%, of the variation in PWV [64, 138]. ESRD results in more advanced vascular morphological alterations, including increased arterial wall thickness and stiffness,

as well as arterial calcification. Mechanisms and progression rate in ESRD therefore go beyond what can be explained by traditional cardiovascular risk factors [62, 64]. In a community-based population like MDCS there are only a few individuals exhibiting this phenotype of arterial remodeling. At re-examination, only 25 individuals (0.9% of the study population) had an eGFR below 30 ml/min/1.73m² (CKD stages 4 or 5), which explains the non-significant results between eGFR and arterial stiffness after adjustment presented in **Paper 3**.

Adrenomedullin

In **Paper 1**, positive cross-sectional relationships between MR-proADM and brachial PP, cIMT and carotid atherosclerotic plaque were shown. The results remained after adjusting for conventional cardiovascular risk factors. This suggests the existence of an association between MR-proADM and arterial stiffness, of which PP is a marker. It also suggests an association with atherosclerosis of which cIMT and carotid plaques are markers. However, MR-proADM was not able to predict c-f PWV at the MDCS-CV re-examination after full adjustment.

Results from previous studies that investigate the relationship between ADM and arterial stiffness are contradictory [139-143]. One previous study, with an aim similar to the aim in **Paper 1**, showed a relationship between MR-proADM and PP in a population of African-Americans, but not in non-Hispanic whites [139].

The fact that MR-proADM did not predict c-f PWV after follow-up may be explained by the fact that ADM is thought to be secreted as an acute compensatory mechanistic factor and might therefore have less long-term predictive properties. To test this, it would be interesting to test ADM and PWV for a cross-sectional relationship. Unfortunately, the two variables were never measured at the same time in MDCS. However, a study from the American Framingham population cohort including 1962 individuals, found no significant association between MR-proADM and c-f PWV [140]. Since ADM is secreted by the endothelium in response to hemodynamic stress, it might be less involved in the changes of the arterial tunica media measured as arterial stiffness by PWV.

The relationship between MR-proADM and cardiovascular risk markers shown in **Paper 1**, combined with previous studies showing a cardiovascular beneficial role of ADM, fits with the hypothesis that the elevation of ADM is probably reflecting compensatory effects [79, 144]. Following stratification for hypertension, MR-proADM was associated with both cIMT and carotid plaque score in hypertensive individuals, but not in normotensive individuals. This could be due to an increased and/or different role of ADM in the clinical setting of hypertension and overt CVD.

Diabetes and hyperglycemia

In **Paper 3**, FPG, HOMA-IR, and HbA_{1c} were all predictive markers of arterial stiffness. FPG was associated with arterial stiffness in cross-sectional analyses. Some of the results from **Paper 3** point towards the harmful effects of impaired glucose metabolism even in the pre-diabetic stage or the normoglycemic range. First, the 2h glucose after OGTT, available only at MDCS-CV re-examination in the population without diabetes, was associated with arterial stiffness in cross-sectional analyses. Secondly, c-f PWV did not differ significantly between individuals with diabetes and individuals meeting the criteria of diabetes at re-examination but with no clinically diagnosed diabetes. Although, this could be due to a more intensified pharmacological and non-pharmacological treatment of cardiovascular risk factors in these individuals. Third, the cross-sectional relationship between FPG and c-f PWV existed throughout the range of FPG, below the threshold for diabetes (<7 mmol/l) and IFG (<6.1 mmol/l) [145].

These results from **Paper 3** are concordant with previously published results showing cross-sectional relationships between IFG and IGT, on the one hand, and with arterial stiffness on the other [56-59]. Data from the Whitehall II study showed that FPG, 2 h glucose and HOMA-IR were predictive of c-f PWV in men but not in women after 16-years of follow-up [126]. That study, however, included a female population (n=912) that was substantially smaller than the male population (n=2857).

It is not clear whether hyperglycemia or insulin resistance is the underlying cause that links T2D to vascular ageing. In **Paper 3**, the association between arterial stiffness and the triad of hyperglycemia, dyslipidemia, and abdominal obesity supports a role of insulin resistance suggests that insulin resistance plays a role in the acceleration of vascular ageing. On the other hand, baseline HOMA-IR did not show a stronger association with arterial stiffness than fasting glucose. This does not support the hypothesis of insulin resistance being a driving force behind the arterial stiffening process, although HOMA-IR can be regarded as too crude and simple a measure to evaluate insulin sensitivity, and ideally the hyperinsulinemic, euglycemic clamp should be used.

Markers of local abdominal arterial stiffness

The markers of regional arterial stiffness demonstrated in **Paper 3** differ to some extent from the results in **Paper 2**, which investigates markers of arterial stiffness locally in the abdominal aorta. This could be due to either a true difference in risk markers or methodological variances. However, the risk markers should not be

drastically different as c-f PWV is a measure of regional arterial stiffness and therefore also includes the stiffness of the abdominal aorta.

Results from **Paper 2** show a markedly higher arterial stiffness in men compared to women. In comparison, the sex differences in c-f PWV presented in **Paper 3** are much smaller. Previous studies using β -stiffness index have also found that men have a much stiffer abdominal aorta than women [146, 147]. Such differences using echo-tracking method could be caused by differences in body composition. With abdominal obesity being more common among men than women, this could complicate the ultrasound measurement in these individuals and introduce a systematic error. On the other hand, it could also reflect true differences between the sexes where male aortas would be stiffer, or stiffen earlier in life, than female aortas.

In **Paper 2**, an association between baseline HOMA-IR and β -stiffness index in women was found that did not exist among men. Such gender differences have also been shown in individuals with type 1 diabetes using an echo-tracking system where diabetic women, but not men, showed higher abdominal aortic stiffness compared with controls [148]. Also, some previous studies using PWV have reported greater associations between diabetes and arterial stiffness in women than in men [149-151]. This has led to the hypothesis that female elastic arteries would be more susceptible to the damaging effect of impaired glucose metabolism than male aortas [56]. However, such differences could not be demonstrated using c-f PWV in **Paper 3**. Waist circumference in men was positively associated with aortic stiffness in **Paper 2**. Associations between β stiffness index and obesity have been previously reported in a study that also demonstrated a reduced aortic stiffness following weight loss from bariatric surgery [152].

Risk factors of vascular ageing

Epidemiological, observational studies are subject to many potential biases including confounding factors and reverse causality. Without being able to prove causality, the results from **Papers 1-3** cannot identify *risk factors*, only *risk markers*. Although important as such, it is not sufficient for a complete pathophysiological understanding and, by extension, pharmacological treatment, including future drug target candidates. As previously discussed, MR is one way to address this issue.

In the results from **Paper 4**, GRSs for FPG and T2D were associated with arterial stiffness, which suggests causal relationships. When using multivariable inverse-variance weighted MR to correct for bias due to pleiotropic effects, β coefficients for T2D SNPs were not associated with arterial stiffness, while the significant

results for FPG SNPs remained. The significant results for T2D from the individual GRS method is interpreted as an effect of pleiotropy, violating the basic assumption of MR. This is further supported by the results from the T2D41 GRS analysis showing that T2D GRS lost its significance for association with c-f PWV after removing seven SNPs also included in the FPG GRS.

The association between GRS for T2D and arterial stiffness was previously investigated in a MR study of 11,385 individuals from Shanghai, China [153]. The results showed a significant positive relationship (OR=1.24, p=0.008) between arterial stiffness measured by b-a PWV and a GRS for T2D including 34 SNPs. These results concur with the results from the individual GRSs in **Paper 4**. To my knowledge, there are no previous publications investigating the relationship between T2D SNPs and arterial stiffness using inverse-variance weighted MR.

Several other studies have shown associations between FPG and arterial stiffness without the presence of T2D. Such results include observations from **Paper 3** and previous publications showing cross-sectional relationship between IFG and arterial stiffness [57-59]. Previous studies have also demonstrated that HbA_{1c} is a predictor of arterial stiffness without the presence of T2D. In fact, HbA_{1c} was related to c-f PWV progression rate over a four years follow-up period in a population without diabetes [154]. Also, HbA_{1c} has been shown to predict arterial stiffness in young individuals with type 1 diabetes [146].

The results from **Paper 4** support the role of the hyperglycemia as the pathophysiological mechanism behind the observed relationship between disturbed glucose metabolism and arterial stiffness, even in the non-diabetic population. Even though an increased FPG is one of the established criteria for T2D, the genetic predisposition of T2D is likely to incorporate several other complex metabolic traits including insulin resistance, lipid turnover, and abdominal fat accumulation. On the contrary, the SNPs associated with fasting glycaemia included in **Paper 4** were established in a population without diabetes and should reflect genetic variations of plasma glucose levels within the normal range, where there is either no tendency or a weaker tendency of progression to T2D. Interestingly, a recent study using MR showed that FPG-increasing genetic variants, not associated with risk of T2D, increased the risk coronary artery disease [155]. Whether arterial stiffness links the two entities together or not is merely speculations but either way the result supports the role of hyperglycemia itself as a cardiovascular risk factor.

Strengths and limitations

The community-based MDCS-CV cohort, with a relatively high number of participants, is a significant strength of the studies included in this thesis. The cohort has been well characterized and results have shown that the baseline cohort was fairly representative of the general population at the baseline examination, even though mortality rates were higher in non-participants [95]. However, at re-examination, the study population was elderly (mean age 72 years), thus resulting in a higher prevalence of co-morbidity and widespread use of pharmacological BP and lipid-lowering treatment. Although this is adjusted for in the multiple regressions, it is still likely to affect the results. Furthermore, the drop-out rate between baseline and follow-up resulted in survival bias, with a significantly healthier population taking part in the re-examination [98]. In **Paper 2**, using the HOMA cohort, subjects with an impaired glucose metabolism were deliberately overrepresented in this study compared with the total MDCS-CV population. This makes it easier to reach significant results with regard to the influence of glucose metabolism but, on the other hand, impairs the external validity.

As arterial stiffness progresses throughout life, the long period of time that elapsed between baseline and follow-up is another advantage. Unfortunately, c-f PWV and β stiffness index were both measured only at follow-up, which ruled out the ability to study the progression of arterial stiffness during the follow-up period.

Typically, in MR, one single SNP is used as the IV. However, since one SNP explains very little of a trait variance, in **Paper 4**, GRSs were used as IVs instead of individual SNPs in order to increase statistical power. This method introduces a risk of pleiotropic effects, thus potentially violating a basic assumption of an IV in MR. The inverse-variance weighted meta-analysis approach is one way to address this issue. In **Paper 4**, no associations were found between arterial stiffness and GRSs for BMI, SBP, LDLc, HDLc, or triglycerides. However, because of the widespread use of lipid and BP-lowering drug therapy in the study population combined with a relatively small study population, in a context of genetic analyses, there is a risk of falsely retaining the null hypothesis. The negative findings from this study should therefore be interpreted with caution and, subsequently, the conclusions from the study focus on the positive findings. Recent GWASs have revealed more SNPs for the cardiometabolic traits investigated in **Paper 4** and the GRSs could be expanded by the addition of these new SNPs [156, 157]. These newly discovered SNPs, however, generally do not add very much to the explanation of the traits. Furthermore, an increased numbers of SNPs to the GRSs could also increase the risk of pleiotropy.

All information about medical history and pharmacological treatment was retrieved from a self-administered questionnaire, which is a weakness. The ultrasound

investigation with assessment of cIMT and plaques in **Paper 1** is an observer-dependent investigation that results in both intra- and inter-observer variability. The echo-tracking method used in **Paper 2** is also observer-dependent. Unfortunately, there is no data available on intra- or inter-observer variability for the β stiffness index from the HOMA-cohort. Pressure exerted on the abdomen while performing the echo-tracking measurements could impair the normal expansion of the aorta during systole and affect the results. Also, brachial BP instead of aortic BP was used in the formula for calculating the β stiffness index. This has been shown to underestimate PP by approximately 10 mmHg, although this underestimation should be similar across the population [147]. The measurement of c-f PWV in **Paper 3** and **Paper 4** is a less user-dependent investigation and represents the current gold standard for measurement of arterial stiffness, an important strength [6]. Furthermore, as the stiffness of the arterial wall is dependent on the loading of stiff components in the arterial wall, the adjustment for MAP is crucial in the effort to isolate the intrinsic elastic properties of the arterial wall [22, 23].

Clinical perspectives

Hypertension represents a major global public health problem resulting in a considerable disease burden and is estimated to cause 7.5 million deaths annually worldwide, corresponding to 12.8% of total mortality [158]. Arteriosclerosis is a central mechanism underlying the increase in SBP seen in advancing age [9]. Although the awareness of hypertension and its treatment is widespread in the medical community, the concept of arteriosclerosis is still not very well established. Arteriosclerosis is often confused with atherosclerosis, and their risk factors and markers are often estimated together [51]. It is important to study the relationship with arteriosclerosis of both classical and non-classical cardiovascular risk factors and risk markers. For example, statins, which have well-documented, positive effects on atherosclerosis, have no, or very small, effects on arteriosclerosis [159].

An understanding of the mechanisms behind the ageing of the arterial wall is central to the evaluation and treatment of cardiovascular risk factors, especially in the growing elderly population. The association between impaired glucose metabolism and arteriosclerosis is an important mechanism of how hyperglycemia could damage the vascular system, which later results in increased risk of cardiovascular events. A recent intervention study found that controlling BP slowed down the progression of arterial stiffness among non-diabetics whereas no such results could be found among individuals with diabetes [160]. The results suggest that, among diabetics, BP control alone may not be sufficient to slow the arterial stiffening process. Interestingly, large-scale clinical trials such as LEADER, [161] SUSTAIN-

6 [162] (both using GLP-1 analogs vs. placebo) and EMPA-REG OUTCOME [163] (using an SGLT2 inhibitor vs. placebo) have reported beneficial effects of plasma glucose-lowering treatment on cardiovascular events and mortality. A reduction of stiffness in the central arteries is one interesting potential explanation behind these results. The observation that arterial stiffness increase early on, before the onset of diabetes, is an important observation as it promotes early interventions.

The role of ADM in the pathogenesis of CVD is yet to be fully understood. It currently has no clinical role as a marker of atherosclerosis or arteriosclerosis, or for the prediction of cardiovascular events. On the other hand, it has shown promising result in other fields, such as being a biomarker for the prognosis and treatment of sepsis [164], as well as a potential target for the treatment of malignant tumors and myocardial infarction [165].

The aged population that participated in the re-examination of MDSC-CV can definitely, and rightfully, be questioned for being a result of survival bias and subject to the heavy influence of pharmacological treatment. On the other hand, the aged population, often already undergoing pharmacological drug treatment, is also at high risk of cardiovascular events. Therefore, studies on vascular ageing should not exclude these kinds of populations.

Erratum

In **Paper 1**, Table 2, the number of individuals with hypertension should read 3196.

Conclusion

- Adrenomedullin is a marker of atherosclerosis measured as carotid intima-media thickness and carotid plaques. It is also a marker of arteriosclerosis measured as pulse pressure.
- Insulin resistance (measured as HOMA-IR) and low HDLc were both predictors of local, abdominal aortic stiffness among women. These relationships were not seen among men.
- Hyperglycemia, dyslipidemia (high triglycerides, low HDLc), and increased waist circumference are all independent non-hemodynamic, long-term predictors of regional arterial stiffness, in both men and women. Smoking, LDLc, and eGFR were not associated with arterial stiffness. As such, the well-known cluster of risk factors for atherosclerosis (smoking, hypertension, LDLc, diabetes) is only partly associated with arterial stiffness.
- Single nucleotide polymorphisms raising fasting plasma glucose is associated with arterial stiffness. However, no association was found between arterial stiffness and single nucleotide polymorphisms raising systolic blood pressure, BMI, LDLc, HDLc, triglycerides, or type 2 diabetes. This suggests that fasting glycaemia, rather than type 2 diabetes, is causally associated with arterial stiffness in the population.

Future perspectives

The interest in arterial stiffness and vascular ageing has grown rapidly during the last decades. Despite recent advances, many questions still remain. Apart from the role of traditional cardiovascular risk markers influencing arterial stiffness, vascular ageing is also believed to be accelerated by less characterized risk factors such as inflammation, early life factors, oxidative stress and telomere length [13].

MR is an important tool for establishing causal effects. The findings in **Paper 4** suggest that FPG, but not T2D, is causally related to arterial stiffness are interesting, but also difficult to interpret. They need replication in future studies from different populations.

Today, arterial stiffness measured as PWV is not examined routinely in clinical practice when evaluating patients for primary or secondary prevention treatments. Will this change in the future? A large number of studies have shown that c-f PWV is a marker of the cumulative damage of risk factors to the arterial wall, thereby showing proof of concept [68]. Two meta-analysis have demonstrated that arterial stiffness is able to predict cardiovascular events and mortality [47, 48]. Furthermore, c-f PWV is also able to re-classify subjects from an intermediate cardiovascular risk into a higher or lower cardiovascular risk category [68]. According to current guidelines by the European Society of Cardiology and European Society of Hypertension, the measurement of c-f PWV adds value to the stratification of individuals at intermediate cardiovascular risk [166]. However, to date, no randomized, controlled trial has yet assessed c-f PWV as a target for pharmacological therapy. In France, the SPARTE study is a currently ongoing intervention study that randomly delegates individuals to either a treatment strategy based on evaluation of arterial stiffness, or to a conventional guideline-driven therapeutic strategy [167]. If an arterial stiffness-based strategy would result in better clinical outcomes, it would further increase the rationale for measuring c-f PWV in clinical practice. Lastly, in order to fully incorporate c-f PWV into clinical practice, this measurement must also be cost-effective.

The implementation of c-f PWV into clinical practice could also be a useful tool to help physicians explain and motivate the patient to start or intensify lifestyle improvements or pharmacological treatment. The concept of an accelerated ageing process and the fact that c-f PWV corresponds to real morphological changes might make arterial stiffness a beneficial treatment target.

As previously stated, there has been a remarkable increase in the interest in vascular ageing during the last decade as evidenced by published and ongoing study cohorts from around the globe [168]. In Malmö, the Malmö Offspring Study (MOS), is an ongoing (as of March 2017) study cohort investigating the children and grandchildren of the MDCS-CV participants. In MOS, c-f PWV is one of the investigated variables, as well as 24-hour central hemodynamics. Using this material, it is hopefully possible to reach a deeper understanding of vascular ageing where, for example, lifestyle and early life factors are two aspects that require more research. Another approach that might be fruitful is to specifically study parts of the population with “abnormally” healthy and compliant arteries for their age. Some individuals with obesity or diabetes, for example, do not have an accelerated arterial stiffness. With a specific focus on these individuals, it may be possible to identify factors that protect the arteries from stiffening [169].

Antihypertensive treatment, especially inhibitors of the Renin-Angiotensin-Aldosterone-System (RAAS) are known to reduce arterial stiffness beyond the acute BP-lowering effect *per se* [159]. A desirable feature of future drugs would be to reduce the stiffness of the arterial wall without any primary effect on BP. One such pathway could be through the selective Angiotensin II type 2 receptor agonist called compound 21 (C21). According to results from studies in rats, this compound prevents aortic stiffening and collagen accumulation with no pronounced effect on BP lowering [170]. Much remains to be discovered about the molecular mechanisms involved in arterial stiffness and to develop new, potential drug targets, more knowledge is necessary. Future studies including genetic variants and gene expression data will hopefully continue to shed new light on such mechanisms.

Populärvetenskaplig sammanfattning på svenska

(Summary in Swedish)

Hjärt-kärlsjukdom är den vanligaste dödsorsaken både i världen och i Sverige. I Sverige orsakar dessa sjukdomar nästan 40 procent av alla dödsfall. Högt blodtryck är en av de viktigaste riskfaktorerna för hjärt-kärlsjukdom och beräknas idag förekomma hos c:a 2 miljoner individer i Sverige. Förekomsten av högt blodtryck i befolkningen stiger kraftigt i takt med ökande ålder.

När vi blir äldre sker förändringar i de stora artärernas kärlväggar, framförallt i stora kroppspulsådern, som gör att artärerna blir styvare. Detta kallas vaskulärt åldrande. Den ökade artärstyvheten gör att det trycket i artärerna då hjärtat pumpar ut blod – det systoliska blodtrycket – stiger. Fenomenet ska inte förväxlas med åderförkalkning (ateroskleros) som innebär en inlagring av bland annat fett och kalk på artärernas insida. Åderförkalkning och artärstyvhet är båda viktiga orsaker till hjärt-kärlsjukdom.

Avhandlingens syfte var att kartlägga vilka riskfaktorer som har samband med och/eller kan förutsäga det vaskulära åldrandets hastighet. Detta har gjorts med hjälp av befolkningsstudien Malmö Kost Cancer (MKC). Malmö Kost Cancer startades, som namnet antyder, för att studera sambandet mellan kost och cancer. En del av deltagarna, c:a 6100 individer, har man dock undersökt ytterligare för att även studera hjärt-kärlsjukdom. Deltagarna undersöktes första gången mellan 1991 och 1996. En del av dessa individer har också undersökts igen 1999-2000 och/eller 2007-2012.

Artärstyvhet kan mätas på flera olika sätt. Den metod som är mest erkänd är att mäta hur snabbt tryckvågen från hjärtat fortplantar sig i artärträdet, så kallad pulsvågshastighet, en relativt enkel och smärtfri undersökning. Man kan också använda sig av ultraljud för att undersöka hur mycket kärlet expanderar i en specifik artärdel då hjärtat slår och med hjälp av detta beräkna styvheten.

I **delarbete 1** undersöktes en signalmolekyl vid namn adrenomedullin, som bland annat bildas i blodkärlen. Resultaten visar att adrenomedullin har samband med

både åderförkalkning och blodtryck. Sammanvägt med tidigare forskning verkar adrenomedullin utsöndras i ökad grad då blodkärlen påfrestas och skulle i framtiden kunna komma att användas som en markör för dessa tillstånd.

I **delarbete 2** och **delarbete 3** undersöktes hur riskfaktorer för hjärtkärlsjukdom kunde prediktera artärernas stelhet vid uppföljande undersökning. Som mått på artärstyvhet användes i **delarbete 2** ultraljud och i **delarbete 3** pulsvågshastighet.

Resultaten från **delarbete 3** visar att vissa välkända riskfaktorer för åderförkalkning, såsom rökning och LDL-kolesterol ("det onda kolesterolet"), inte har någon koppling till artärernas styvhet. Dock såg man att bland annat ökat midjemått, diabetes och lågt HDL-kolesterol ("det goda kolesterolet") var kopplat till artärstyvhet vid uppföljning. Vidare fanns det även ett samband mellan artärstyvhet och blodsocker hos individer utan diagnostiserad diabetes vilket tyder på att artärerna kan skadas redan under förstadiet till diabetes. I **delarbete 2** sågs samband mellan känslighet för hormonet insulin och stora kroppspulsåderns styvhet hos kvinnor men inte hos män. Det kan tolkas som att kvinnors kärl är mer känsliga än mäns, men resultatet ska tolkas med försiktighet på grund av en del metod-svagheter i detta delarbete.

En utmaning i befolkningsstudier är att bevisa äkta samband, så kallad kausalitet. Med det menas att avgöra huruvida statistiska samband, såsom de som sågs i **delarbetena 1-3**, verkligen orsakas av ett äkta orsakssamband eller om de samvarierar av andra orsaker. I **delarbete 4** användes en metod som kallas Mendelsk randomisering där man använder sig av genetiska markörer som inte påverkas av de störfaktorer som annars finns i befolkningsstudier, t.ex. livsstil eller läkemedelsbehandling. Det viktigaste resultatet från **delarbete 4** var att det genetiska uttrycket för förhöjt fastebloodsocker har samband med artärstyvhet. Studien pekar därmed på att blodsockret i sig bidrar till att kärlen åldras snabbare. Vidare forskning inom detta område skulle i framtiden kunna leda till att det vaskulära åldrandet kan bromsas eller till och med reverseras med tidig prevention hos riskindivider.

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