Mechanisms of cardiovascular disease in the metabolic syndrome and type 2 diabetes mellitus : focus on adverse intermediate phenotypes

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**Mechanisms of cardiovascular disease in the metabolic
syndrome and type 2 diabetes mellitus:
focus on adverse intermediate phenotypes**

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PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
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Voor mijn ouders

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The background of the entire page is a close-up, top-down view of a wooden surface covered with numerous white, heart-shaped cookies. The cookies vary in size and are scattered across the frame, creating a textured, patterned effect. The wood grain is visible beneath the cookies.

Chapter 1

Introduction

Introduction

The incidence of type 2 diabetes mellitus (DM2) is rapidly increasing, partially due to population growth, aging and a western lifestyle, leading to an increase in the prevalence of obesity (1). In 2007 almost 750,000 diagnosed individuals with diabetes mellitus were registered at the Dutch general practitioners' database, of which over 90% had DM2 (2). In the coming years, this number will increase to an estimated number of 1.3 million around 2025. Obviously, this will lead to a heavy burden on the health care system, as DM2 is associated with increased morbidity and mortality. Cardiovascular disease (CVD) accounts for over 60% of the DM2 associated morbidity and mortality (3-5).

'Traditional' cardiovascular risk factors, such as obesity, dyslipidaemia or hypertension often characterize individuals with DM2. These risk factors possibly interact with each other and other factors although such interactions are not easy to quantify (6). A hypothetical model of the interactions among some of these 'traditional' and 'non-traditional' risk factors is shown in **Figure 1**, where one can appreciate that while some of these factors tend to cluster, others appear to be independent of each other. Most of them may actually be common antecedents for both DM2 and CVD, supporting the hypothesis that both DM2 and CVD arise separately from a "common soil" (7). Consistent with this hypothesis, some of these 'non-traditional' cardiovascular risk factors predict incident DM2 (8, 9). Low-grade inflammation, endothelial dysfunction, and abnormalities of coagulation are all associated with (central) obesity and insulin resistance and may thereby become common antecedents of both DM2 and CVD.

In the prediabetic stage the clustering of risk factors is better known as the metabolic syndrome (MetS). This is a construct defined by the coexistence of central obesity, dyslipidaemia, elevated blood pressure, and impaired glucose metabolism. Overall, MetS is associated with approximately a 5-fold increased risk of incident DM2 (10), a 2-fold increase in risk of CVD, CVD mortality and stroke, and a 1.5-fold increase in risk of all-cause mortality (11).

Several definitions for MetS have been proposed of which the ones from the National Cholesterol Education Programme Adult Treatment Panel III (NCEP-ATP III) and International Diabetes Federation (IDF) are the most widely used (12, 13). These definitions use the same set of traits for diagnosing MetS, i.e., impaired glucose metabolism, hypertension, central obesity, high triglycerides and/or low high-density lipoprotein (HDL) cholesterol, only differing by the fact that the IDF definition required central obesity plus any 2 other traits, whereas NCEP required any 3 out of 5 traits. In

2009, NCEP and IDF agreed that, for uniformity reasons, central obesity was not an obligatory component anymore for the diagnosis of MetS (14).

Figure 1. Interaction of risk factors in the pathogenesis of cardiovascular disease. Adapted from Fonseca et al (6)

Nevertheless, central obesity remains a key factor at the root of the association between MetS and DM2 on the one hand and CVD on the other, as (central) obesity is both associated with incident DM2 (15, 16) and CVD (17, 18). Although the pathophysiological mechanism(s) linking (central) obesity to CVD are not completely understood, it is known that an increase in adipose tissue elicits pathophysiological processes which are involved in the development of atherosclerosis, such as low-grade inflammation, insulin resistance, hypertension and dyslipidaemia. Adipose tissue is an active endocrine and paracrine organ that releases a large number of cytokines and adipokines, such as leptin, adiponectin, interleukin-6 (IL-6) and tumour necrosis factor- α (TNF- α), that affect not only body weight homeostasis, but also affect insulin resistance, glucose metabolism, lipid levels, blood pressure, coagulation, fibrinolysis, low-grade inflammation and atherosclerosis (19).

Epidemiological studies investigating associations between (central) obesity and cardiovascular outcomes generally use anthropometric estimates of (central) obesity, such as body-mass index (BMI), waist circumference and waist-to-hip-ratio (WHR). These measures are not always an accurate reflection of a person's true amount of fat mass (and distribution). For example, in the young, BMI does not distinguish between fat and lean

(non-fat) mass and in the elderly, lean mass decreases which can result in a paradoxically unchanged or decreased BMI despite an unchanged or even increased fat mass (20). Sophisticated imaging techniques, such as magnetic resonance imaging (MRI), computed tomography (CT) and dual-energy absorptiometry (DXA), are able to distinguish between fat and lean masses in the total body and in specific body regions, thereby enabling a more precise appreciation of the metabolic and cardiovascular risks associated with body fat distribution (20, 21).

Surrogate markers of CVD

The use of surrogate markers of CVD is very common in epidemiological research. This is due to the fact that for most clinical research questions, the expected event rate of cardiovascular events is low resulting in the need for a large sample size and long duration of follow-up to obtain adequate statistical power (22). A surrogate marker is defined as a characteristic that can objectively be measured and evaluated as an indicator of normal biologic or pathogenic processes or as a physiologic response to a therapeutic intervention (23). The criteria for a useful surrogate marker are efficiency (i.e. the surrogate marker should be relatively easy to evaluate), linkage (i.e. the quantitative and qualitative relationship between the surrogate marker and the clinical endpoint should be established on the basis of epidemiological and clinical studies), and congruence (the surrogate should produce parallel estimates of risk and benefits that are related to the target disease processes as endpoints) (23, 24). Increased levels of these surrogate markers potentially detect individuals with an unfavourable intermediate phenotype, i.e. those who have a higher risk for future cardiovascular events. In this thesis we will mainly focus on the following intermediate phenotypes of CVD: endothelial dysfunction, microalbuminuria, cardiovascular autonomic dysfunction (C-AD), arterial remodeling and thrombin generation. As mentioned above, these adverse intermediate phenotypes may help us to give insight in the biological process and identify individuals with a higher risk of CVD, which could have implications for treatment and prevention programmes.

Endothelial dysfunction

Endothelial dysfunction is thought to be one of the first events in the process leading to atherosclerosis and is a good predictor of future cardiovascular events (25-27). Normal functions of endothelial cells include decreasing vascular tone, maintaining vascular permeability and limiting coagulation, platelet adhesion and aggregation (28). Endothelial dysfunction is thought to be present when its properties, either in the basal state or after

stimulation, have changed in a way that is inappropriate with regard to the preservation of organ function. It is commonly associated with deficiencies of nitric oxide (NO) and prostacyclin bioavailability. A closely related event is binding of mononuclear cells, such as monocytes and T-lymphocytes, to the endothelium, which is mediated by adhesion molecules present on the endothelial surface, such as soluble vascular cell adhesion molecule (sVCAM-1), soluble intercellular adhesion molecule (sICAM-1) and soluble endothelial selectin (sE-selectin). This process leads to foam cell and in a later stage to plaque formation.

Endothelial function can be assessed both biochemically and functionally and possibly by microalbuminuria (28). Biochemical assessment can be done by measuring biomarkers, such as sVCAM-1, sICAM-1, Von Willebrand factor and sE-selectin. Functional assessment measures the ability of a blood vessel to dilate after a stimulus (e.g. ischaemia-induced shear stress) (**Figure 2**). Brachial artery flow-mediated vasodilation (FMD) is a noninvasive method of assessing arterial endothelial function *in vivo*. Because endothelial injury is an early event in atherogenesis, it has been suggested that an abnormal FMD may precede the development of structural changes in the arterial wall. It is known that FMD is impaired in subjects with DM2 (29, 30). The metabolic syndrome and higher levels of body fatness, particularly a central pattern of fat distribution, have been inversely associated with FMD (31-35). However, these findings were restricted to relatively young individuals and whether these results can be generalized to older individuals is unknown. Furthermore, the potential role of peripheral lean mass has not been studied.

Figure 2: An example of a flow-mediated dilation curve by Pyke et al(36) --- shear rate, — diameter.

Microalbuminuria and autonomic dysfunction

Microalbuminuria is a strong independent risk marker for CVD in both diabetic (37, 38) and non-diabetic individuals (39, 40). Microalbuminuria is defined as an urinary albumin excretion of 20-200 $\mu\text{g}/\text{min}$, or 30-300 $\text{mg}/24$ hour (41). There are several mechanisms by which microalbuminuria could be associated with CVD (42). It is unlikely that microalbuminuria itself causes cardiovascular mortality (i.e. that microalbuminuria is a risk factor for CVD). A more plausible explanation is that microalbuminuria and CVD have a common underlying risk factor or pathophysiological process(es). For example, endothelial dysfunction precedes and predicts the onset of microalbuminuria, e.g. by an increased glomerular pressure and vasopermeability, and is also associated CVD. Cardiovascular autonomic dysfunction could potentially also be one of the explaining factors behind the microalbuminuria-CVD relationship (43, 44) as it is associated with both microalbuminuria and CVD (vide infra).

Cardiovascular autonomic dysfunction refers to dysfunction of the non-voluntary, non-sensory nervous system affecting mostly the internal organs, including the cardiovascular system (45, 46). Common symptoms of C-AD are disturbances of the heart rate, such as resting tachycardia, orthostatic hypotension and an inadequate increase of the heart rate during exercise. Furthermore, damage to the autonomic nerves can decrease symptoms of coronary artery disease and thus lead to silent myocardial infarction (47, 48). Cardiovascular autonomic dysfunction is independently associated with microalbuminuria, which could potentially be explained by a reduced nightly drop in blood pressure and/or a disturbance in glomerular arteriolar autoregulation; (43) in addition, C-AD is independently associated with cardiovascular mortality (**Figure 3A**) (49, 50). Thus, C-AD could, in part, explain the risk of CVD associated with microalbuminuria. Alternatively, it is also possible that both microalbuminuria and C-AD are associated with cardiovascular mortality by different biological pathways (**Figure 3B**). The role of autonomic dysfunction in the association between microalbuminuria and cardiovascular mortality has up till now not been investigated in a prospective setting.

Figure 3A. Cardiovascular autonomic dysfunction as a risk factor for both microalbuminuria and cardiovascular mortality. **3B.** Microalbuminuria and cardiovascular autonomic dysfunction are associated with cardiovascular mortality by independent biological mechanisms. -----> non-causal relationship —> causal relationship

Arterial remodeling

Increased intima-media thickness (IMT) is an early marker of atherosclerosis and has independently been associated with CVD (51, 52). Traditionally, research on ischaemic stroke risk mainly focused on carotid IMT and relatively little attention was paid to variations in arterial diameter accompanying wall thickening. Arterial remodeling is the structural change of the blood vessel in response to changes in blood flow and circumferential stretch to restore normal shear stress and wall tension, respectively (53-55). It is characterized by wall thickening (indicated by an increase in IMT) and diameter widening (indicated by an increase in interadventitial diameter (IAD)).

Three different patterns of arterial remodeling have been identified (**Figure 4**): inward (characterized by a decrease in lumen diameter (LD) resulting from a greater change in IMT than in IAD), outward (characterized by an increase in LD caused by a greater change

in IAD than in IMT), and compensatory (characterized by LD preservation despite changes in IMT and IAD) (54).

Maladaptive remodeling (i.e. an increase in tensile stress despite increases in IMT and IAD) may be associated with stroke as it has been shown that in ischaemic stroke wall tension was higher at the affected side of the carotid artery (56). Individuals with DM2 have been characterized with a maladaptive pattern of remodeling of the carotid artery (57). As mentioned above, patients with MetS, but without DM2, are still at high risk for CVD mortality and stroke (11), a phenomenon which is better known as the ‘ticking clock’ hypothesis (58). It is thus of interest to ascertain the extent to which MetS is already associated with (maladaptive) remodeling of the carotid artery, which would be in line with the hypothesis that damage already occurs long before DM2 becomes overt. To better understand the dynamic process of arterial remodeling, an investigation of carotid structural and functional changes as function of changes in MetS status over time in the context of a longitudinal study is needed.

Figure 4: Different patterns of arterial remodeling

Hypercoagulability / thrombin generation

DM2 is associated with a prothrombotic state, i.e. increased platelet activation and aggregation (primary haemostasis), increased activation of the coagulation cascade (secondary haemostasis), decreased fibrinolysis (in part due to upregulation of plasminogen activator inhibitor-1 (PAI-1)) (59). Thrombin is a key enzyme of secondary haemostasis by combining pro- and anticoagulant functions through controlled regulation of the cascade activity (60, 61). DM2 has been associated with thrombin activation markers (prothrombin fragments 1.2 and thrombin-antithrombin complexes TAT) (62-64). However, given the complexity of the coagulation system, which involves several interrelated procoagulant and anticoagulant pathways, measuring the plasma levels and/or activities of individual proteins is of limited usefulness in epidemiological studies and clinical management of hypo- and hypercoagulable states.

Therefore, an automated method (Calibrated Automated Thrombogram, CAT) that quantitatively measures thrombin generation *in vitro* in plasma after activation of the coagulation cascade with tissue factor (TF), phospholipids and calcium chloride (CaCl_2) has been developed (65). This method generates a thrombin generation curve that mimics the overall plasma coagulability potential when a thrombogenic stimulus (e.g. TF release after plaque rupture) appears (**Figure 5**). This curve is characterized by a lag (initiation) phase, followed by a thrombin burst (propagation), which is then eventually completely inhibited by plasma protease inhibitors (termination). The endogenous thrombin potential (ETP) is reflected by the area under this curve, which represents the total amount of active thrombin that is formed after activation of the coagulation cascade.

ETP is thought to provide a more reliable estimation of coagulation under pathophysiological conditions than the single point measurement of activation markers such as prothrombin 1.2 fragments or TAT complexes (67). Furthermore, ETP has been shown to be a good indicator of a hypercoagulable state in, for example, studies among individuals using oral contraceptives, prior venous thromboembolism or circulating lupus anticoagulans (65, 68-70). Up till now, only one small case-control study has investigated the association between DM2 and thrombin generation according to this method (71). No studies have been performed at the population level. Establishing such an association is a necessary step to ascertain its potential value as a tool to estimate atherothrombotic risk in individuals with impaired glucose metabolism (IGM) and/or DM2. Furthermore, from an aetiological point of view, it is not clear whether any such association is independent of other risk factors that typically characterize these individuals (e.g. hypertension and

central adiposity) or, alternatively, is explained by these risk factors and their associated pathophysiological mechanisms.

Figure 5: An example of a thrombin generation curve obtained by means of the CAT method in normal individuals. The parameters derived from the thrombin generation curve are: lag time (the time in which ~ 10 nM of thrombin is formed), peak height (the maximum amount of formed thrombin), and ETP (the area under the curve). *From: van Veen et al. (66)*

Adipose tissue also has both direct and indirect effects on coagulation (72). Direct effects are for example synthesis of tissue factor and PAI-1 by adipose tissue, whereas indirect effects are mainly mediated by effects of adipose tissue-derived factors, such as leptin and adiponectin, on platelet activation and increased secretion of proinflammatory cytokines by the liver which in turn affect coagulation. A recent pilot study in children has shown that extreme obesity is associated with increased plasma-based thrombin generation (73). No studies have investigated the association between body fat mass and thrombin generation according to the CAT method in adults. In addition, the extent to which different regions of body fat and lean mass contribute to or protect against a hypercoagulable state as estimated by this method are also unknown.

Study population

In this thesis we made an attempt to further study the above mentioned surrogate markers, or indeed, intermediate phenotypes, for CVD, by making use of the data of the Hoorn Study and the Amsterdam Growth and Health Longitudinal Study.

The Hoorn study is a Dutch population-based cohort study on glucose metabolism and its complications which started in 1989 and consisted of 2484 participants (74). All participants were of Caucasian origin and between 50 and 75 years old at the time of inclusion. All subjects had a 75-g oral glucose tolerance test (OGTT), except those diagnosed previously with diabetes, as indicated by treatment with oral glucose-lowering drugs or insulin (**Chapter 3**).

Between 1996 and 1998, a 2nd examination (1st follow-up) was carried out. From the initial cohort, 150 people had died and 108 had moved out of Hoorn before 1996. One hundred forty other people were not invited because of logistical reasons. Of the remaining 2086 people who were invited for the follow-up examination, 1513 (72.5%) participated (75).

In 2000–2001, a 3rd examination (2nd follow-up) was carried out among surviving participants who gave their permission to be recontacted (**Chapters 2,4,6,7**). All participants who had diabetes, as determined by an oral glucose tolerance test (OGTT) or by diabetes treatment (n=176), at the 1st follow up visit were invited. In addition, a random sample of participants who had normal glucose tolerance (n=705) or impaired glucose tolerance (n=193) at the 1st follow up visit were invited. Of the 1074 individuals invited, 648 (60.3%) participated. The main reasons for nonparticipation in the 2000–2001 follow-up examination were lack of interest (30%) or comorbidity (23%). Other reasons were high age (7%), unwillingness to travel (6%), participation considered too time-consuming (6%), miscellaneous reasons (15%) and 13% gave no reason (76). At this follow-up examination the DXA (body composition) measures, arterial properties, thrombin generation parameters and biomarkers of endothelial function were measured.

Amsterdam Growth and Health Longitudinal Study (AGAHLS). For **Chapter 5** we used data derived from the AGAHLS, an observational longitudinal study that started in 1976 with a total inclusion of 698 boys and girls (mean age of 13.1 ± 0.8 years) (77). Briefly, its initial goal was to describe the natural development of growth, health and lifestyle of adolescents, and to investigate life-course relationships between biological and lifestyle

risk factors. In the year 2000 (8th measurement round), when subjects' mean age was 36.6 (± 0.6) years, measurements of large artery properties by means of non-invasive ultrasound were included for the first time in this cohort, and obtained in 373 subjects (78). In 2006, complete follow-up of arterial measurements were obtained in 297 of these individuals (79).

Objectives of this thesis

Specifically, we formulated the following research questions:

1. Is an adverse body composition, i.e. more central fat mass, but also less peripheral fat and/or lean mass associated with endothelial dysfunction, as ascertained by FMD and/or by biomarkers and could any association be explained by body-composition-related levels of low-grade inflammation and/or insulin resistance?
2. Is the association between microalbuminuria and (cardiovascular) mortality explained by cardiovascular autonomic dysfunction?
3. Is MetS in non-diabetic individuals associated with maladaptive remodeling of the carotid artery?
4. Are changes in carotid artery structural and functional properties related to changes in MetS-status in a 6-yr longitudinal study in otherwise healthy young adults?
5. Is total body fat mass associated with hypercoagulability as estimated by plasma-based thrombin generation and what is the role of different body regions in this respect?
6. Are impaired glucose metabolism and DM2 independently associated with thrombin generation in plasma and to what extent can this association be explained by traditional risk factors?

Outline of this thesis

In **Chapter 2** the association between body composition and endothelial dysfunction was addressed and the potential mediating role of low-grade inflammation and/or insulin resistance were analysed. The role of cardiovascular autonomic dysfunction in the association between microalbuminuria and cardiovascular mortality was examined in **Chapter 3**. In **Chapter 4** we investigated whether MetS in non-diabetic individuals was associated with maladaptive remodeling of the carotid artery; subsequently, the longitudinal association between MetS and carotid artery properties in young adults was

investigated in **Chapter 5**. We explored the relation between body composition and thrombin generation, and investigated which regional body fat depots contributed to this association in **Chapter 6**. In addition, we examined the potential mediating role of low-grade inflammation, insulin resistance and adiponectin. In **Chapter 7**, we focused on the association between glucose metabolism status and thrombin generation and investigated whether any such association could be attributable to ‘traditional’ cardiovascular risk factors. Finally, in the general discussion (**Chapter 8**) methodological and pathophysiological issues are presented and implications and suggestions for future studies are addressed.

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Chapter 2

Higher central fat mass and lower peripheral lean mass are independent determinants of endothelial dysfunction in the elderly. The Hoorn Study.

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Abstract

Objective - To investigate first, the extent to which an adverse body composition is associated with ED; and, second, whether any such associations could be explained by low-grade inflammation (LGI) and/or insulin resistance (HOMA2-IR).

Design - We studied 475 individuals from the Hoorn Study (mean age, 68.9±6.1 years, 245 women). Body composition was assessed by whole body dual-energy absorptiometry. Endothelial dysfunction was measured functionally, by flow-mediated dilation (FMD) and by circulating biomarkers (summarized into an ED score, with a higher score indicating more ED). Associations were examined with multiple linear regression models and mediation analyses according to the ab product of coefficients method.

Results - After adjustment for age, sex, glucose metabolism status, prior cardiovascular disease and lifestyle factors, total and central fat mass were positively associated with the ED score [$\beta=0.16$ (95% CI 0.03; 0.28) and $\beta=0.16$ (0.03; 0.29) respectively] and inversely, although not statistically significantly, with FMD_{max}. Peripheral fat mass was not associated with the ED score or FMD_{max}. There was a significant favourable association between peripheral lean mass and FMD_{max} [$\beta=0.15$ (0.00; 0.30)], but not with the ED score. The association between total and central fat mass and the ED score was, to a great extent, mediated by LGI and HOMA2-IR. In contrast, LGI or HOMA2-IR did not mediate the association between peripheral lean mass and FMD_{max}.

Conclusion - Higher levels of central, but not peripheral fat mass were adversely associated with ED, which was attributable to body-composition-related LGI and insulin resistance. Peripheral lean mass was favourably associated with endothelial function, suggesting that sarcopenia may also play a role in the decline of endothelial function in elderly individuals.

Introduction

Endothelial dysfunction (ED), whether measured functionally (flow-mediated dilation - FMD) or by determining the levels of circulating biomarkers, such as soluble vascular cell adhesion molecule 1 (sVCAM-1), soluble intercellular adhesion molecule 1 (sICAM-1), Von Willebrand factor (vWF) and soluble endothelial selectin (sE-selectin), is associated with cardiovascular disease (CVD) (1-4).

A decline in endothelial function is evident with advancing age (5-7). Age-related changes in body composition, which are typically characterized by increases in body fatness (8, 9), may play a role herein. Indeed, numerous studies have linked excessive body fat to ED [reviewed in (10)] and pathobiological mechanisms such as obesity-related low-grade inflammation (LGI) and insulin resistance, all of which are intertwined, have been proposed to explain such an adverse link (10-13). However, only few studies have been performed in older individuals (14, 15) and most, if not all, have focused mainly on the association of total or central body fatness with ED (11, 14-21). The extent to which peripheral fat mass correlates with ED is unclear. Evidence suggests that peripheral body fat may actually be protective against metabolic and cardiovascular risk factors (22-26) and disease (27), but whether this could reflect favourable associations with ED needs to be examined. Also, the potential beneficial role of lean mass has not been taken into account. Indeed, after middle age, changes in body composition are typically characterized not only by increases in fat mass but also by decreases in lean mass (8, 9). Sarcopenia, i.e. the loss of skeletal muscle and function with ageing, which may be a marker of poor nutrition and/or physical activity levels, is a major contributor to frailty in older adults, but may also affect ED and thereby cardiovascular health as well through mechanisms such as inflammation and/or insulin resistance (28). Studies examining the associations between lean mass and measures of ED are lacking, however.

In view of these considerations, we investigated, in a population-based cohort of elderly individuals: first, the extent to which an adverse body composition, i.e. more central fat mass but also less peripheral fat and/or lean mass is associated with ED, as ascertained by FMD and/or by circulating biomarkers; and, second, whether any such associations could be explained by body-composition-related levels of LGI and/or insulin resistance.

Materials & Methods

Subjects and study design

The Hoorn Study is a cohort study on glucose tolerance and CVD in a general Caucasian population of 50-75 years old (n=2484), which started in 1989 as has been described extensively before (25, 29, 30). Follow-up examinations of this cohort were performed in 1996–1998 and 2000–2001 when arterial properties, including a full FMD protocol (by means of non-invasive ultrasonography) and body composition (by means of dual-energy absorptiometry (DXA)) were measured for the first time (25, 31). For this follow-up examination, all participants who had diabetes, as determined by a 75-g oral glucose tolerance test, or by treatment (n=176) (29), and random samples of participants who had normal glucose tolerance (n=705) or impaired glucose metabolism (n=193) at the 1996–1998 examination were invited. Of the 1074 individuals invited, 648 (60.3%) participated in the 2000–2001 follow-up examination; main reasons for non-participation were lack of interest (30%) or comorbidity (23%); other reasons included high age (7%), unwillingness to travel (6%), participation considered too time-consuming (6%) and miscellaneous reasons (15%), and 13% gave no reason (25). For the present study we excluded 173 of these 648 individuals due to missing data on DXA measurements (n=25), incomplete (i.e. only baseline diameter) or qualitatively unsatisfactory ultrasound examinations (n=158) and/or no assessments of circulating markers of ED (n=12). These individuals were older and had a more adverse risk factor profile than the 475 included in this study [Supplemental Table 1 – online only material].

The Ethical Review Committee of the VU University Medical Centre approved the study protocol and all participants gave their written informed consent.

Body composition

Individuals underwent a whole body DXA scan using the fan beam technology (QDR-2000, software version 7.20D, Hologic, Brussels, Belgium) to obtain measures of lean tissue, fat and bone mineral masses for the total body and for standard body regions. Regions of the head, trunk, arms and legs were distinguished with the use of specific anatomic landmarks (25). In the analyses we used fat and lean soft tissue masses from the trunk, arms and legs. Peripheral fat mass was calculated by adding the fat mass of the legs to that of the arms, and peripheral lean mass was calculated by adding the lean mass of the legs to that of the arms (22, 23).

Endothelial dysfunction

Brachial artery flow-mediated dilation (FMD) and nitroglycerin-mediated dilation (NMD)

Individuals underwent an ultrasound examination of FMD and NMD according to the guidelines of the International Brachial Artery Reactivity Task Force (32). The measurement protocol has been described in detail previously (31, 33). Briefly, baseline diameter (mean of three measurements) was assessed with the use of an ultrasound scanner equipped with an 7.5 MHz linear probe (350 series, Pie Medical, Maastricht, The Netherlands) that was connected to a PC equipped with vessel wall movement detection software and an acquisition system (Wall Track System, Pie Medical, Maastricht, The Netherlands). Baseline peak flow velocity (mean of two measurements) was determined by pulsed-wave Doppler from a sample volume in the centre of the artery at a 60° angle. A pressure cuff, placed on the forearm, was then automatically inflated and kept constant at supra-systolic pressure (SBP) (brachial SBP +100mmHg) in order to induce forearm ischemia. After 5 min the cuff was released leading to an increase in blood flow and hereby in shear stress, which served as the stimulus for FMD response. Maximum peak flow velocity was measured within 15 s after the cuff release, and measures of arterial diameter were subsequently measured at 45, 90, 180 and 300s. Next, 15 min of rest were taken to re-establish baseline conditions. Baseline diameter (mean of 3 measurements) and peak flow velocity (mean of two measurements) were re-determined as described above. Nitroglycerin (400 µg, Nitrolingual Spray, Pohl-Boskamp, Germany) was then administered sublingually; after 5 min, diameter (mean of 3 measurements) and peak flow velocity (mean of 2 measurements) were again determined.

In some individuals measures of brachial diameter were missing at one (n=36), two (n=14) or three (n=7) out of the 4 post cuff release time points. For these individuals, we imputed the missing post cuff release diameter(s) with a longitudinal regression method (34). Specifically, we regressed the repeated post cuff release measures of diameter available on baseline diameter and time with the use of generalized estimating equations. Because the relationship between time and diameter is not linear, time was treated as a categorical (dummy) variable to approximate the FMD response curve by estimation of the predicted diameter at each exact time point (i.e. at 45, 90, 180 or 300s after cuff release) based on the observed baseline and post cuff-release measures obtained (35). The predicted values hereby estimated were used to replace the missing value(s). Peak FMD (FMD_{max} , in mm) was then calculated as maximum post-occlusion diameter – baseline diameter and was used in the analyses as main dependent variable. NMD (in mm), that is, endothelium-independent vasodilation thought to reflect smooth muscle cell function, was calculated as diameter at 5min post nitroglycerin ingestion - baseline diameter.

Circulating markers of endothelial dysfunction and low-grade inflammation

Levels of sICAM-1, sVCAM-1, sE-selectin, C-reactive protein (CRP), serum amyloid A (SAA), interleukin 6 (IL-6), interleukin 8 (IL-8) and tumour necrosis factor- α (TNF- α) were assessed in serum samples by a multi-array detection system based on electro-chemiluminescence technology [MesoScaleDiscovery (MSD), SECTOR Imager 2400, Gaithersburg, Maryland, USA] (36, 37). In addition, levels of vWf and of CRP were (also) determined in citrated plasma by means of ELISA, as described in detail elsewhere (36). All these markers were standardized to common units by transformation into z-scores [i.e. (individual value–population mean)/population SD] and aggregated into: an overall circulating markers of endothelial dysfunction score (hereafter referred to as ED score), by averaging the z-scores of sICAM-1, sVCAM-1, sE-selectin and vWf; and an overall circulating markers of LGI score, by averaging the z-scores of CRP (mean of $zCRP_{MSD}$ and $zCRP_{ELISA}$), SAA, IL-6, IL-8, TNF- α and sICAM-1. Because sICAM-1, is expressed by both monocytes and the endothelium (38) it may be included in both overall scores (36, 37).

Covariates

Health status, medical history, medication use, and smoking, dietary (including alcohol consumption) and physical activity habits were assessed by questionnaires (30, 39). Levels of fasting plasma glucose, insulin, estimated glomerular filtration rate (eGFR), total, HDL- and LDL-cholesterol, triglycerides, brachial systolic blood pressure and diastolic blood pressure were measured/estimated as described elsewhere (29, 30, 40). Insulin resistance was calculated by means of the web-based Homeostasis Model of Assessment (HOMA2-IR) (41) and subjects' glucose metabolism status according to the WHO 1999 definition (42).

Statistical analyses

All analyses were performed by using the Statistical Package for Social Sciences (SPSS) version 18.0 (SPSS Inc., Chicago, IL). Comparisons of the clinical characteristics across tertiles of the main outcome variables (ED score or FMD_{max}) were investigated with analysis of variance (ANOVA) for continuous variables and chi-square test for frequency data. Variables with a skewed distribution, i.e. triglycerides, CRP, IL-6, IL-8, SAA, HOMA2-IR and the physical activity score were loge transformed prior to the analyses.

We used multiple linear regression models to investigate the extent to which, first, total body fat%, and second, regional body composition (i.e. trunk fat, peripheral fat and peripheral lean mass, mutually adjusted for each other) were independently associated with the ED score and/or FMD. These analyses were adjusted for age, sex, glucose metabolism status, prior CVD and lifestyle risk factors (i.e., smoking habits, physical

activity, total energy intake and alcohol consumption) as potential confounders (model 1). Additional adjustments included baseline diameter and/or increments in peak systolic velocity (models 2-4), in analyses with FMD_{max} as dependent variable only. The adjustment for baseline diameter was done to enable comparison with other studies. It should be noticed, however, that such adjustment may not be appropriate in the context of the present study (observational cohort), because an assumption of equal baseline diameters between groups of individuals does not hold (as in randomized controlled trials); this adjustment may thus lead to an overestimation of the strength of the associations when the study determinants are positively associated with baseline diameter, which in turn is known to be inversely associated with FMD_{max} (43).

In the model with body composition as main determinant we excluded trunk lean mass because of its very high correlation with peripheral lean mass (Pearson's $r > 0.945$), which would disturb the stability of the regression analyses when included in the models due to multi-collinearity (tolerance < 0.1).

Mediating role of insulin resistance and/or low-grade inflammation

The mediating role (i.e. extent and significance) of variable(s) reflecting pathobiological mechanisms hypothesized to be in the pathway between a given determinant (X) and outcome (Y) was ascertained by quantitative appreciation of the attenuation in the strength of the association (β) between X and Y after adjustment for the potential mediator(s) (M) (44, 45). Accordingly, we used the 'ab product of coefficients' method (45) to investigate the mediating role of LGI (M1) and/or HOMA2-IR (M2) in the associations described above. These analyses entail a series of steps, the details of which are explained and illustrated in **Figure 1** (see also legend). To ascertain statistical significance by any of the mediating 'effects' we used the SPSS macros provided by Preacher & Hayes to compute bootstrapped (bias-corrected) confidence intervals (5,000 samples) for the size of the specific mediation 'effect' by M1 and/or by M2, hereby accounting for the distribution of the ab products which is often skewed (46). These analyses enabled the appreciation of whether any of the proposed mediators exerted a predominant role in the associations investigated, thus contributing to a better understanding of potential operative pathophysiological mechanisms (44-46).

All results are shown as standardized regression coefficients (β) and their respective 95% confidence intervals (CIs). A two-sided probability value of < 0.05 was considered statistically significant.

Results

Table 1 shows general characteristics, as well as body composition, biomarkers of LGI, HOMA2-IR, biomarkers of ED and the brachial artery properties and FMD_{max} of the individuals included in this study (n=475), stratified by tertiles of the ED score or FMD_{max}. In general, the cardiovascular risk profile was worst at the most adverse levels of the ED score and/or FMD_{max}, i.e. at their highest or lowest tertiles, respectively.

Some risk factors seemed to be specifically associated with the ED score (i.e. male sex, weight, insulin, HOMA2-IR, total-to-HDL cholesterol ratio, triglycerides and trunk fat mass) or FMD (height, total calorie intake, peripheral lean mass). The ED score and FMD_{max} were inversely and significantly, though modestly, correlated with each other (Pearson's $r = -0.156$, $p < 0.01$).

Total body fat, regional body composition and endothelial dysfunction

Total body fat % was positively and significantly associated with the ED score [$\beta = 0.16$ (95% CI 0.03; 0.28)] and inversely, although not statistically significant, with FMD_{max} (**Table 2**, model 1).

When the contribution of body composition to ED was examined in more detail, we observed a significant adverse association between trunk fat and the ED score [$\beta = 0.16$ (95%CI 0.03; 0.29)], but not with FMD_{max} [$\beta = -0.04$ (-0.17; 0.13)] and a significant favourable association between peripheral lean mass and FMD_{max} [$\beta = 0.15$ (0.00; 0.30)], but not with the ED score. In contrast to trunk fat, peripheral fat mass was not associated with the ED score [$\beta = -0.03$ (-0.17; 0.12)] and, similarly to trunk fat, peripheral fat was not associated with FMD_{max} [$\beta = -0.02$ (-0.17; 0.13)] – **Figure 2**.

Adjustments for baseline diameter and Δ PSV. Despite the inverse association between baseline diameter and FMD_{max} [$\beta = -0.16$ (-0.28; -0.04)], adjustments for baseline diameter did not affect the magnitude of the associations between total fat % or trunk or peripheral fat masses and FMD_{max} (**Table 2**, models 2) since none of these were significantly associated with baseline diameter. The associations between peripheral lean mass and FMD_{max} became stronger after adjustments for baseline diameter [$\beta = 0.23$ (0.08; 0.39)], which was explained by a positive association between peripheral lean mass and baseline diameter [$\beta = 0.76$ (0.66; 0.86)]. Adjustment for Δ PSV did not affect any of the associations mentioned above (**Table 2**, models 3).

Figure 1. Illustration of a simple (panels A and B) and a multiple mediation models (panels A and C), in the context of the present study.

Panel A shows the association of an independent variable X (i.e. body fat % or trunk fat, peripheral fat or peripheral lean masses) on a dependent variable Y (i.e. ED score or FMD_{max}), after adjustments for age, sex, glucose metabolism status, prior CVD, smoking status, physical activity, daily energy intake and alcohol consumption (covariates); path c is represented by a standardized regression coefficient (β), and represents total 'effect' of X on Y.

Panel B illustrates how the role of a single mediator (M) is assessed in simple mediation analyses. Mediation is considered to be present when the strength of the β between X and Y (path c) decreases by a nontrivial amount after further adjustment for a M [i.e. low-grade inflammation (LGI score) or insulin resistance (HOMA2-IR)]; this figure thus represents both the direct 'effect' of X on Y (path c') and the indirect 'effect' of X on Y through M, the later being quantified by the product of the 2 paths linking X to Y through that mediator (path $a * \text{path } b$ or ab). These paths are quantified by β s retrieved from two separate regression models including all covariates, one

examining the association between X and M (path a) and the other the association between M and Y independently of also X (path b).

Panel C summarizes how the role of two mediators (M1 and M2) is quantified in a multiple mediation analyses; this figure thus represents both the direct effect of X on Y (path c') and the indirect effect of X on Y through each of the Ms. The specific indirect effect of X on Y through M1 is defined by the product of the two paths linking X to Y through that mediator, i.e., a_1b_1 ; similarly, the specific indirect effect of X on Y through M2 is defined as a_2b_2 . Paths a_1 and a_2 are estimated as in simple mediation, but paths b_1 and b_2 now also include mutual adjustment for each other. The total indirect effects of X on Y is the sum of the two specific indirect effects, i.e., $a_1b_1 + a_2b_2$; The total effect of X on Y is thus the sum of the direct effect and all of the indirect effects: $c = c' + (a_1b_1 + a_2b_2)$. ED score: circulating biomarkers of endothelial dysfunction score; FMD_{max} : flow-mediated dilation; LGI score: low-grade inflammation score; HOMA2-IR: insulin resistance derived from the homeostasis model assessment.

Table 1. General characteristics of the study population according to tertiles (T) of the circulating markers of endothelial dysfunction (ED) score or maximal flow-mediated dilation (FMD)

Characteristics	Circulating markers of endothelial dysfunction (ED) score				Flow-mediated dilation (FMD)			
	T1 (n=158)	T2 (n=159)	T3 (n=158)	p-trend	T1 (n=158)	T2 (n=159)	T3 (n=158)	p-trend
Age, years	67.6 ± 5.6	68.2 ± 5.9	71.0 ± 6.3	<0.001	71.7 ± 7.0	68.4 ± 5.5	66.7 ± 4.5	<0.001
Sex, % men	43	49	53	0.072	50	43	53	0.653
Height, cm	169 ± 9	170 ± 9	170 ± 9	0.733	169	168	171	0.004
Weight, kg	75 ± 12	77 ± 11	79 ± 12	0.006	77 ± 12	75 ± 11	78 ± 12	0.721
Body mass index, kg/m ²	26.1 ± 3.2	26.6 ± 3.2	27.3 ± 3.6	0.001	27.1 ± 3.7	26.5 ± 3.2	26.3 ± 3.1	0.035
Fasting glucose, mmol/l	5.8 ± 0.7	6.2 ± 1.6	6.5 ± 1.5	<0.001	6.4 ± 1.4	6.2 ± 1.6	5.8 ± 0.9	<0.001
Insulin, pmol/l	48 [35-66]	54 [39-73]	70 [48-100]	<0.001	57 [43-80]	54 [39-82]	54 [40-75]	0.251
Insulin resistance (HOMA2-IR)	0.94 [0.67-1.27]	1.04 [0.77-1.43]	1.36 [0.98-1.98]	<0.001	1.11 [0.84-1.55]	1.04 [0.76-1.62]	1.05 [0.77-1.48]	0.158
Use of glucose-lowering medication, %	4	10	13	0.010	14	10	3	0.028
Glucose metabolism status, % NGM/IGM/DM2	61/26/13	56/23/21	31/35/34	<0.001	39/28/34	52/27/22	58/30/12	<0.001
Total-to-HDL cholesterol ratio	4.1 ± 1.1	4.3 ± 1.2	4.5 ± 1.4	0.001	4.4 ± 1.3	4.2 ± 1.3	4.3 ± 1.1	0.547
Triglycerides, mmol/l	1.20 [0.90-1.70]	1.30 [0.90-1.70]	1.40 [1.00-1.80]	0.005	1.30 [1.00-1.80]	1.30 [0.90-1.70]	1.30 [1.00-1.70]	0.103
Use of lipid-lowering medication, %	9	15	16	0.072	20	12	8	0.003
Systolic blood pressure, mm Hg	136 ± 17	140 ± 20	147 ± 19	<0.001	147 ± 19	141 ± 19	135 ± 17	<0.001
Diastolic blood pressure, mm Hg	74 ± 8	75 ± 9	78 ± 10	0.002	78 ± 10	76 ± 9	74 ± 8	<0.001
Use of antihypertensive medication, %	22	33	39	0.002	49	25	20	<0.001
eGFR, ml/min/1.73m ²	82 ± 15	79 ± 17	78 ± 16	0.070	78 ± 18	82 ± 16	80 ± 14	0.404
Total energy intake (kcal/day)	2008 ± 1027	1944 ± 446	1947 ± 585	0.466	1893 ± 518	1939 ± 573	2067 ± 992	0.033
Ever smoker, %	56	58	65	0.098	67	56	55	0.039
Alcohol intake, % none/ mod/high ^a	13/53/34	11/37/59	23/48/30	0.052	19/53/29	15/54/32	13/54/34	0.128
Physical activity score (/1000)	6.18 [3.72-8.68]	4.38 [2.76-7.20]	4.66 [2.19-7.38]	<0.001	4.05 [1.98-6.59]	5.44 [3.17-8.63]	5.37 [3.44-8.07]	<0.001
Prior cardiovascular disease, %	43	43	58	0.007	60	50	35	<0.001
Body composition by DXA								
Total body fat, %	34 ± 9	34 ± 9	35 ± 9	0.127	35 ± 9	35 ± 9	33 ± 9	0.014
Trunk fat mass, kg	12.2 ± 4.9	13.0 ± 4.8	14.4 ± 5.2	<0.001	13.8 ± 5.2	13.1 ± 4.7	12.7 ± 5.1	0.045
Trunk lean mass, kg	24.0 ± 4.8	24.6 ± 4.6	25.0 ± 4.7	0.063	24.4 ± 4.8	24.0 ± 4.6	25.2 ± 4.7	0.096
Peripheral fat mass, kg	11.5 ± 3.8	11.8 ± 4.1	12.0 ± 4.4	0.283	12.0 ± 4.3	11.9 ± 3.9	11.4 ± 4.2	0.202
Peripheral lean mass, kg	19.4 ± 4.9	19.5 ± 4.4	19.7 ± 4.6	0.607	19.3 ± 4.6	18.8 ± 4.5	20.5 ± 4.6	0.018

Circulating markers of low grade inflammation (LGI)								
Tumour necrosis factor α (ng/l)	7.8 $\pm$ 2.5	9.2 $\pm$ 3.1	9.7 $\pm$ 2.8	<0.001	8.9 $\pm$ 2.2	8.6 $\pm$ 3.0	9.1 $\pm$ 3.4	0.622
Interleukin-8 (ng/l)	13.3 [10.4- 17.4]	15.0 [11.5-18.8]	16.2 [12.8-21.7]	<0.001	15.5 [12.3-20.2]	14.5 [11.7-17.9]	14.5 [11.0-19.7]	0.138
Interleukin-6 (ng/l)	1.25 [0.95-2.01]	1.40 [1.00-1.92]	1.75 [1.27-2.95]	<0.001	1.69 [1.20-2.48]	1.43 [1.02-2.13]	1.33 [0.96-2.05]	0.015
C-reactive protein _{ELISA} (mg/l)	1.21 [0.63-2.33]	1.85 [1.23-3.55]	3.16 [1.31-6.13]	<0.001	2.16 [1.16-4.44]	1.85 [1.05-3.42]	1.56 [0.82-3.68]	0.013
C-reactive protein _{MSD} (mg/l)	1.23 [0.64-2.59]	2.05 [1.35-3.96]	3.30 [1.56-6.52]	<0.001	2.47 [1.23-5.04]	1.96 [1.02-3.79]	1.71 [0.94-3.69]	0.022
Serum amyloid A (mg/l)	1.39 [0.85-2.45]	1.66 [1.08-2.73]	2.09 [1.16-4.42]	<0.001	1.78 [1.10-3.13]	1.56 [0.96-3.07]	1.71 [1.00-2.78]	0.549
LGI score	-0.36 $\pm$ 0.44	-0.04 $\pm$ 0.45	0.45 $\pm$ 0.73	<0.001	0.13 $\pm$ 0.63	0.01 $\pm$ 0.66	-0.09 $\pm$ 0.65	0.003
Circulating markers of endothelial dysfunction (ED)								
Soluble intercellular adhesion molecule 1 (μ g/l) ^b	-	-	-	-	268 $\pm$ 59	251 $\pm$ 56	242 $\pm$ 57	<0.001
Soluble vascular adhesion molecule 1 (μ g/l)	-	-	-	-	413 $\pm$ 91	403 $\pm$ 98	389 $\pm$ 75	0.014
sE-selectin (μ g/l)	-	-	-	-	19.8 $\pm$ 9.6	19.0 $\pm$ 7.6	18.1 $\pm$ 6.7	0.071
von Willebrand Factor (%)	-	-	-	-	164 $\pm$ 54	148 $\pm$ 47	148 $\pm$ 46	0.003
ED score	-	-	-	-	19.9 $\pm$ 74.4	-0.28 $\pm$ 70.9	-12.4 $\pm$ 59.4	<0.001
Flow-mediated dilation (FMD)								
<u>Diameter</u>								
Baseline (μ m)	4545 $\pm$ 699	4681 $\pm$ 801	4684 $\pm$ 721	0.096	-	-	-	-
Maximum (μ m)	4741 $\pm$ 701	4853 $\pm$ 788	4838 $\pm$ 725	0.244	-	-	-	-
Absolute change (μ m)	197 $\pm$ 125	172 $\pm$ 133	154 $\pm$ 133	0.004	-	-	-	-
Relative change (%)	4 $\pm$ 3	4 $\pm$ 3	3 $\pm$ 3	0.002	-	-	-	-
<u>Peak Systolic Velocity</u>								
Baseline (cm/s)	55 $\pm$ 12	59 $\pm$ 13	60 $\pm$ 15	0.002	-	-	-	-
After reactive hyperaemia (cm/s)	105 $\pm$ 27	108 $\pm$ 27	106 $\pm$ 24	0.752	-	-	-	-
Absolute change (cm/s)	50 $\pm$ 23	49 $\pm$ 24	46 $\pm$ 19	0.117	-	-	-	-
Relative change (%)	94 $\pm$ 44	87 $\pm$ 49	82 $\pm$ 37	0.009	-	-	-	-
Nitroglycerin-mediated dilation								
Baseline diameter (μ m)	4571 $\pm$ 683	4697 $\pm$ 806	4720 $\pm$ 727	0.083	-	-	-	-
Maximum diameter (μ m)	4997 $\pm$ 700	5085 $\pm$ 799	5089 $\pm$ 728	0.282	-	-	-	-
Absolute change (μ m)	464 $\pm$ 220	409 $\pm$ 178	413 $\pm$ 241	0.040	-	-	-	-

Data are means $\pm$ SD, frequencies (%) or medians [inter-quartile range]. NGM, normal glucose metabolism status; IGM, impaired glucose metabolism status; DM2, type 2 diabetes; HOMA2-IR, Homeostasis Model Assessment of insulin resistance

^a defined as: 0 glasses/day (none), 1-2 glasses/day in men or 1 glass/day in women (moderate) and >2 in men or >1 glasses/day in women (high).

^b included in both the ED score as the LGI score

Figure 2. Associations between total body fat % (single bar) and regional body composition (clustered bars), measured by DXA, with the following markers of endothelial dysfunction: **A**, circulating markers of endothelial dysfunction (ED score); **B**, maximal brachial flow-mediated dilation (FMD_{max}). All data are adjusted for age, sex, glucose metabolism status, prior CVD, smoking status, physical activity, daily energy intake and alcohol consumption; clustered bars reflecting the independent associations of trunk fat, peripheral fat and peripheral lean masses are also mutually adjusted for each other.

Table 2. Associations between body composition and endothelial dysfunction

Main determinants	Model	Adjustments	Main study outcomes			
			ED score		FMD _{max}	
			β	95%CI	β	95% CI
Total body fat %	1	Age, sex, GMS, lifestyle RFs ^b , prior CVD	0.16[†]	0.03; 0.28	-0.09	-0.21; 0.04
	2	Model 1 + baseline diameter	-	-	-0.09	-0.21; 0.04
	3	Model 1 + Δ PSV	-	-	-0.08	-0.21; 0.04
	4	Model 1 + baseline diameter & Δ PSV	-	-	-0.09	-0.21; 0.04
Trunk fat mass ^a	1	Age, sex, GMS lifestyle RFs ^b , prior CVD	0.16*	0.03; 0.29	-0.04	-0.17; 0.09
	2	Model 1 + baseline diameter	-	-	-0.04	-0.17; 0.08
	3	Model 1 + Δ PSV	-	-	-0.04	-0.17; 0.09
	4	Model 1 + baseline diameter & Δ PSV	-	-	-0.04	-0.17; 0.08
Peripheral fat mass ^a	1	Age, sex, GMS lifestyle RFs ^b , prior CVD	-0.03	-0.17; 0.12	-0.02	-0.17; 0.13
	2	Model 1 + baseline diameter	-	-	-0.01	-0.15; 0.14
	3	Model 1 + Δ PSV	-	-	-0.02	-0.17; 0.13
	4	Model 1 + baseline diameter & Δ PSV	-	-	-0.01	-0.15; 0.14
Peripheral lean mass ^a	1	Age, sex, GMS lifestyle RFs ^b , prior CVD	-0.09	-0.25; 0.06	0.15*	0.00; 0.30*
	2	Model 1 + baseline diameter	-	-	0.23[†]	0.08; 0.39
	3	Model 1 + Δ PSV	-	-	0.15*	0.00; 0.30*
	4	Model 1 + baseline diameter & Δ PSV	-	-	0.23[†]	0.08; 0.39

β are standardized regression coefficients and indicates the change in main outcome variable or potential mediator (in SD) per 1 SD increase in main determinant

* p<0.05, [†] p<0.01, [‡] p<0.001

^amodels are mutually adjusted for the other body composition variables; ^b smoking, total energy intake, alcohol consumption and physical activity;

CVD: cardiovascular disease; ED: endothelial dysfunction; FMD: flow-mediated dilation; GMS: glucose metabolism status; HOMA2-IR: Homeostasis Model Assessment of insulin resistance; LGI: low-grade inflammation; NMD: nitroglycerin-mediated dilation; PSV: peak systolic velocity; RFs: risk factors.

Table 3. Association of total body fat percentage and body composition with the endothelial dysfunction (ED) score or maximal flow-mediated dilation (FMD_{max}) and the (total and independent) mediation role of low-grade inflammation and/or insulin resistance herein

Main determinant	Path	Description	Simple mediation analyses				Multiple mediation analyses		
			By LGI score		By HOMA2-IR		By LGI & HOMA2-IR		Independent mediation (%) ^a
			β	95% CI	β	95% CI	β	95% CI	
Total body fat %	C	Body fat % → ED score	0.16 [†]	0.03; 0.28	0.16 [†]	0.03; 0.28	0.16 [†]	0.03; 0.28	-
	a ₁	Body fat % → LGI score	0.25 [‡]	0.12; 0.38	-	-	0.25 [‡]	0.12; 0.38	-
	a ₂	Body fat % → HOMA2-IR	-	-	0.48 [‡]	0.34; 0.59	0.48 [‡]	0.34; 0.59	-
	b ₁	LGI → ED score	0.57 [‡]	0.50; 0.64	-	-	0.54 [‡]	0.47; 0.62	-
	b ₂	HOMA2-IR → ED score	-	-	0.29 [‡]	0.19; 0.39	0.18 [‡]	0.09; 0.26	-
	c'	Body fat % → Mediator(s) → ED score	0.02	-0.09; 0.12	0.02	-0.11; 0.15	-0.06	-0.17; 0.05	-
	<i>Mediation effects</i>								
	Total indirect association through mediator(s) (c-c')		0.14 [‡]	0.06; 0.23	0.14 [‡]	0.09; 0.21	0.22 [‡]	0.13; 0.32	-
		Specific indirect association through LGI (a ₁ b ₁)	-	-	-	-	0.13 [‡]	0.06; 0.21	59%
		Specific indirect association through HOMA2-IR (a ₂ b ₂)	-	-	-	-	0.09 [‡]	0.05; 0.14	41%
	<i>Comparison of specific indirect associations through LGI vs. HOMA2-IR</i>		-	-	-	-	-0.05	-0.14; 0.04	-
Trunk fat mass	C	Trunk fat → ED score	0.16*	0.03; 0.29	0.16*	0.03 ; 0.29	0.16*	0.03; 0.29	-
	a ₁	Trunk fat → LGI score	0.28 [‡]	0.15; 0.41	-	-	0.28 [‡]	0.15; 0.41	-
	a ₂	Trunk fat → HOMA2-IR	-	-	0.53 [‡]	0.42; 0.63	0.53 [‡]	0.42; 0.63	-
	b ₁	LGI → ED score	0.57 [‡]	0.50; 0.64	-	-	0.54 [‡]	0.47; 0.62	-
	b ₂	HOMA2-IR → ED score	-	-	0.30 [‡]	0.20; 0.41	0.19 [‡]	0.10; 0.28	-
	c'	Trunk fat → Mediator(s) → ED score	0.00	-0.11; 0.11	0.00	-0.13; 0.14	-0.09	-0.20; 0.02	-
	<i>Mediation effects</i>								
	Total indirect association through mediator(s) (c-c')		0.16 [‡]	0.08; 0.24	0.16 [‡]	0.08; 0.24	0.25 [‡]	0.16; 0.35	-
		Specific indirect association through LGI (a ₁ b ₁)	-	-	-	-	0.15 [‡]	0.08; 0.24	60%
		Specific indirect association through HOMA2-IR (a ₂ b ₂)	-	-	-	-	0.10 [‡]	0.05; 0.26	40%
	<i>Comparison of specific indirect associations through LGI vs. HOMA2-IR</i>		-	-	-	-	-0.05	-0.15; 0.03	-
Peripheral fat mass	C	Peripheral fat mass → ED score	-0.03	-0.17; 0.12	-0.03	-0.17; 0.12	-0.03	-0.17; 0.12	-
	a ₁	Peripheral fat mass → LGI score	-0.11	-0.26; 0.04	-	-	-0.11	-0.26; 0.04	-
	a ₂	Peripheral fat mass → HOMA2-IR	-	-	-0.16*	-0.28; -0.03	-0.16*	-0.28; -0.03	-
	b ₁	LGI → ED score	0.57 [‡]	0.50; 0.64	-	-	0.54 [‡]	0.47; 0.62	-
	b ₂	HOMA2-IR → ED score	-	-	0.30 [‡]	0.20; 0.41	0.19 [‡]	0.10; 0.28	-
	c'	Peripheral fat → Mediator(s) → ED score	0.04	-0.08; 0.16	0.02	-0.12; 0.17	0.06	-0.06; 0.18	-
	<i>Mediation effects</i>								
	Total indirect association through mediator(s) (c-c')		-0.07	-0.17; 0.02	-0.05	-0.12; -0.01	-0.09	-0.20; -0.00	-

			Specific indirect association through LGI (a_1b_1)		-	-	-	-	-0.06	-0.16; 0.02	66%
			Specific indirect association through HOMA2-IR (a_2b_2)		-	-	-	-	-0.03	-0.08; 0.01	33%
<i>Comparison of specific indirect associations through LGI vs. HOMA2-IR</i>					-	-	-	-	0.03	-0.05; 0.13	-
<i>Peripheral lean mass</i>	c	Peripheral lean mass $\rightarrow$ FMD _{max}	0.15*	0.00; 0.30	0.15*	0.00 ; 0.30	0.15*	0.00; 0.30	-	-	-
	a ₁	Peripheral lean mass $\rightarrow$ LGI score	-0.23 [†]	-0.29; -0.08	-	-	-0.23 [†]	-0.29; -0.08	-	-	-
	a ₂	Peripheral lean mass $\rightarrow$ HOMA2-IR	-	-	0.12	-0.05; 0.25	0.12	-0.05; 0.25	-	-	-
	b ₁	LGI $\rightarrow$ FMD _{max}	-0.01	-0.10; 0.08	-	-	-0.03	-0.12; 0.06	-	-	-
	b ₂	HOMA2-IR $\rightarrow$ FMD _{max}	-	-	0.10	-0.01; 0.21	0.09	-0.03; 0.20	-	-	-
	c'	Peripheral lean mass $\rightarrow$ Mediator(s) $\rightarrow$ FMD_{max}	0.15	-0.01; 0.30	0.14	-0.01; 0.28	0.13	-0.02; 0.28	-	-	-
<i>Mediation effects</i>											
Total indirect association through mediator(s) (c-c')			0.00	-0.02; 0.03	0.01	-0.01; 0.04	0.02	-0.02; 0.06	-	-	-

β , are standardized regression coefficients

All associations are adjusted for the following covariates: age, sex, glucose metabolism status, prior cardiovascular disease, smoking status, physical activity, daily energy intake and alcohol consumption; b paths include also adjustments for total body fat % or trunk fat, peripheral fat and peripheral lean masses and, in multiple mediation analyses, for other mediator.

* p<0.05, [†] p<0.01, [‡] p<0.001

^a Calculated as proportion of the total indirect effects attributed to the specific mediators: e.g. (0.13/0.22)x100 for LGI and (0.09/0.22x100) for HOMA2-IR in the association between total body fat % and ED score.

Mediating role of insulin resistance and low grade-inflammation

Total body fat % was significantly associated with a higher LGI score [$\beta=0.25$ (0.12; 0.38)] and with HOMA2-IR [$\beta=0.48$ (0.34; 0.59)] (**Table 3**, paths a1 and a2, respectively). In turn, LGI score [$\beta=0.57$ (0.50; 0.64)] and HOMA2-IR [$\beta=0.29$ (0.19; 0.39)] were significantly associated with the ED score (**Table 3**, paths b1 and b2 in simple mediation analyses), even when mutually adjusted for each other and total body fat % [$\beta=0.54$ (0.47; 0.62) and $\beta=0.18$ (0.09; 0.26), paths b1 and b2 in multiple mediation analyses, respectively]. As a result, the association between total fat % and the ED score [$\beta=0.16$ (0.03; 0.28), path c] was strongly attenuated after adjustments for the two mediators [to $\beta=-0.06$ (-0.17; 0.05), path c'] and was no longer significant. The magnitude of the attenuation, or the indirect association through the mediators (paths c-c') was statistically significant [$\beta=0.22$ (0.13; 0.32)] and due to significant and independent contributions by each of the mediators [$\beta=0.13$ (0.06; 0.21) and $\beta=0.09$ (0.05; 0.14), or ~59% and 41%, by LGI and HOMA2-IR, respectively]. The specific mediation effects by LGI or by HOMA2-IR were not statistically different from each another [$\beta=-0.05$ (-0.14; 0.04)].

We found very similar results when the mediating roles of LGI and/or HOMA2-IR in the associations between trunk fat and the ED score were investigated (**Table 3**).

Although peripheral fat mass was not significantly associated with the ED score, LGI and HOMA2-IR nevertheless played a significant mediating effect in this association [$\beta=-0.09$ (-0.20; -0.00)], but paths a1 and a2, reflecting the favourable associations of peripheral fat with LGI and HOMA2-IR [$\beta=-0.11$ (-0.26; 0.04) and $\beta=-0.16$ (-0.28; -0.03), respectively] operated in opposite direction compared to paths b1 and b2, reflecting the adverse associations between these two mediators and the ED score [$\beta=0.54$ (0.47; 0.62) and $\beta=0.19$ (0.10; 0.28, respectively)]. Comparison of the independent mediating effects by LGI [$\beta=-0.06$ (-0.16; 0.02)] and HOMA2-IR [$\beta=-0.03$ (-0.08; 0.01)] showed a tendency for a predominant mediating effect by LGI (i.e. 66 vs. 33% respectively) but this was not statistically significant [$\beta=0.03$ (-0.05; 0.13), **Table 3**].

In contrast, LGI or HOMA2-IR did not seem to play any significant mediating role in the beneficial associations between peripheral lean mass and FMD_{max} (**Table 3**). Indeed, although peripheral lean mass was significantly associated with lower LGI scores [$\beta=-0.23$ (-0.19; -0.08)] it was not with HOMA2-IR [$\beta=0.12$ (-0.05; 0.25)], and neither LGI nor HOMA2-IR were independently associated with FMD_{max} after adjustment for study covariates and body composition [$\beta=-0.03$ (-0.12; 0.06) and $\beta=0.09$ (-0.03; 0.20), respectively]. Therefore, the beneficial association between peripheral lean mass and FMD_{max} [$\beta=0.15$ (0.00; 0.30)] was only very slightly attenuated after adjustments for LGI

and HOMA2-IR [to $\beta=0.13$ (-0.02; 0.28)], and the magnitude of this attenuation was not significant [$\beta=0.02$ (-0.02; 0.06)].

Additional analyses

The results did not differ when the analyses were confined to individuals with complete post-occlusion data only (n=418) (data not shown). Additional adjustment for blood pressure, eGFR, dyslipidemia, use of antihypertensive, lipid-lowering or glucose-lowering drugs did not materially affect any of the associations reported above (data not shown). Because sICAM-1 was included in both the LGI and ED scores, we also ran the mediation analyses excluding sICAM-1 from the LGI score. Results were not materially different from the ones reported in **Table 3** though (data not shown). We have also examined and found no associations between body composition and NMD (data not shown). Finally, the results did not differ between men and women (p-values for interaction all ≥ 0.136).

Discussion

The present study has four main findings. First, and in line with many previous reports, higher levels of total body fat % were associated with ED. Second, when body composition was examined in more detail, we observed that such adverse associations could be attributed to accumulation of fat mass in the trunk, but not in the limbs. Third, we observed independent associations between higher levels of trunk fat mass and of peripheral lean mass with ED, the former was predominantly associated (adversely) with the circulating biomarkers of ED (ED score) and the latter was predominantly associated (favourably) with flow-mediated dilation of the brachial artery (FMD_{max}). Finally, the adverse association of total body fat %, as of trunk fat mass, with the ED score were explained (i.e. mediated), to a similar extent, by related higher levels of low-grade inflammation (LGI score) and insulin resistance (HOMA2-IR), but these two pathobiological mechanisms did not seem to explain the favourable association between peripheral lean mass and FMD_{max}.

To the best of our knowledge, this is the first study that has examined, in an elderly population, the associations of the distribution of body fat and also lean mass (as ascertained by DXA) with ED (as ascertained by the levels of a large panel of biomarkers in the circulation and also by FMD). In addition, we have also explored the extent to which pathways such as LGI and insulin resistance could explain any such associations, and presented these findings in a comprehensive manner, hereby overcoming the problems of otherwise fragmented information in the epidemiological literature available thus far (e.g.

focused on paths a or b or paths c only, without linking the three under a conceptual pathobiological mechanistic model – see **Figure 1**).

Trunk vs. peripheral fat. Our findings are consistent with the concept that excessive adipose tissue, especially when accumulated centrally, has more adverse effects on cardiovascular risk than does adipose tissue stored in peripheral depots. Indeed, it is becoming increasingly clear that peripheral fat depots are metabolically different from fat accumulated centrally (47) and may even carry beneficial effects on metabolic and cardiovascular health (22-26). In support of this view, we observed that peripheral fat mass was not only favourably associated with insulin resistance, but also with LGI, such that these favourable associations seemed to deter, to some extent, the adverse impact of both LGI and insulin resistance on endothelial function, hereby explaining the lack of an adverse impact of peripheral fat on ED.

Peripheral lean mass. It has been postulated that the U-shaped association between BMI and mortality may reflect the opposite effects of lean mass (favourable) and fat mass (adverse) on survival (48). So far, most studies on body composition and ED have been mainly focused on the deleterious effects of excessive fat mass and/or a central pattern of fat distribution (11, 14-21), disregarding the potential beneficial effects of lean mass herein. Our findings indeed suggest that, in elderly individuals, not only higher levels of central fat, but also lower levels of lean mass may be associated with ED. These findings support the view that sarcopenia, i.e. the degenerative loss of skeletal muscle mass and strength associated with aging, could play an important role in the progressive decline of endothelial function and related cardiovascular sequelae with ageing (28). Giving the increasing age of our population and therefore of the prevalence of sarcopenia, our findings have relevant clinical implications by emphasizing the need to carefully monitor losses in lean mass in weight-loss interventions among the elderly. Inclusion of physical exercise in such interventions may be key in this regard (6). In this line it was recently shown that regular exercise improves endothelial function independently of changes in body weight (49). In addition, Haspicova et al have also shown a significant improvement in endothelial responsiveness during an oral glucose load in pre-menopausal obese women who lost 7% of their body weight, but not at the expense of lean mass (50).

Endothelial dysfunction: ED score and FMD_{max} . The term endothelial dysfunction has been most often used to denote impairment of the endothelium-dependent vasodilation capacity, but may be better characterized by encompassing measures of endothelial activation as reflected by abnormalities in endothelial interactions with leukocytes, platelets and regulatory substances as well (51). Indeed, the endothelium actively

regulates vascular tone and permeability, the balance between coagulation and fibrinolysis, the composition of the subendothelial matrix, the adhesion and extravasation of leukocytes, and inflammatory activity in the vessel wall (52). Therefore, there is no ideal single test to (non-invasively) measure endothelial function, as it has many functions (53, 54) and has many dimensions (55). By examining the association between body composition and both FMD_{max} and the ED score we have thus attempted to capture endothelial dysfunction as comprehensively as possible. This constitutes an advantage when comparing our study with the available literature, commonly reporting on FMD (11, 14-17, 19) or one or few circulating biomarkers (18, 20, 21) only.

Pathobiological mechanisms. Decreased nitric oxide (NO) availability, elevated endothelin-1, insulin resistance, LGI and oxidative stress have been postulated as mechanisms for impaired endothelial function in older adults (6, 28). The associations between total and central fat mass and ED were, indeed, and to a great extent, attributable to LGI and insulin resistance. The beneficial association between peripheral lean mass and ED, however, was not. Other sarcopenia-related factors, such as oxidative stress, could explain this beneficial association (6, 10, 56). It should be noted, however, that, in contrast to adipose tissue, a known source of pro-inflammatory cytokines and trigger of hyperinsulinaemia, both of which are intertwined and known to exert effects on the endothelium, the link between lean mass and ED may not be a causal one but a reflection of common underlying factors, such as physical activity and/or nutrition status, instead (10, 28, 49, 50). Both exercise training and dietary changes (e.g. increased fruit, vegetables intake and omega-3 fatty acids rich product, reduced sodium and saturated fat) have been shown to improve ED through mechanisms such as improved NO availability, increased prostaglandin release, reduced free radical-mediated NO degradation, decreased oxidative stress and lower sympathetic vasoconstrictor tone (10, 28). Although our findings were adjusted for the levels of calorie intake (including alcohol) and physical activity, we can not fully rule out residual confounding due to imprecise and/or insufficient measurement of these factors by means of questionnaires (30, 39).

Our study had some limitations that warrant discussion. First, because of its cross-sectional design, any inferences about causality should be made with caution, and the direction of the pathways proposed may not be exclusive (for instance, insulin resistance may also be a consequence of ED). Second, one assumption of combining biomarkers into an overall score, as we did for the LGI and ED scores, is that each biomarker reflects, to a similar extent, the underlying pathophysiological process. It is however unknown whether this is the case for all of the biomarkers used in this study. The use of an overall score allowed us, however, to circumvent the problem of multiple testing otherwise present

when analyses would be conducted with each of the biomarkers separately. Third, determination of FMD_{max} at a specific time point - most often after 60s of cuff release, as in the original protocol proposed by Celermajer et al (57) – leads to a significant underestimation of its true value, as in some individuals FMD_{max} may occur after this time frame (58). With our FMD protocol, which included repeated measures at 45, 90, 180 and 300 s after cuff release, we were able to circumvent this problem, but only for as much as FMD_{max} could be captured by these time points. More recent automated diameter analysis systems enable continuous measurement of post cuff release diameters at 3-5s time intervals, and thereby a more precise determination of the FMD_{max} (59). Therefore with our protocol we may still have underestimated FMD_{max} . However, this was non-differential and, if anything, led to an underestimation of the associations reported. Fourth, despite that trunk fat mass, as assessed by DXA, correlates highly with intrabdominal fat (60), it does not distinguish between subcutaneous and visceral fat in the trunk/abdominal area. Fifth, bias due to selective attrition (healthier sub-population attending the 2000-2001 examination round and the portion of these included in the present study) could have caused an underestimation the ‘true’ associations examined in our study (61). Finally, we studied a white, elderly population and it remains to be established whether our findings can be generalized to other ethnicities and to younger individuals.

In conclusion, in this study in elderly individuals, higher levels of total and central fat mass, but not peripheral fat mass, were adversely associated with ED, which could be attributable to adipose-tissue-related LGI and insulin resistance. Furthermore, there was an inverse, i.e. beneficial, association between peripheral lean mass and ED, suggesting that sarcopenia may also play an important role in the decline of endothelial function and related sequelae in these individuals.

Disclosure

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ONLINE SUPPLEMENTAL MATERIAL

Table S1. Comparison of the general characteristics and body composition of individuals included vs. excluded from the present study

Characteristics	Excluded (n=173)		Included (n=475)	P-value
	n ^a			
Age, years	173	73.1 ± 6.7	68.9 ± 6.1	<0.001
Sex, % men	173	50	48	0.771
Height, cm	172	168 ± 9.3	169 ± 8.8	0.019
Weight, kg	172	81.4 ± 14.9	76.7 ± 11.8	<0.001
Body mass index, kg/m ²	172	29.0 ± 5.0	26.7 ± 3.4	<0.001
Fasting glucose, mmol/l	171	6.4 ± 1.6	6.2 ± 1.4	0.036
Insulin, pmol/l n	165	65 [43-96]	55 [40-78]	<0.001
Insulin resistance (HOMA2-IR)	165	1.28 [0.81-1.85]	0.94 [0.66-1.28]	<0.001
Use of glucose-lowering medication, %	170	11.2	9.1	0.425
Glucose metabolism status, % NGM/IGM/DM2	164	35/34/31	50/28/22	0.002
Total-to-HDL cholesterol ratio	170	4.3 ± 1.3	4.3 ± 1.3	0.998
Triglycerides, mmol/l	170	1.3 [1.0-1.9]	1.3 [1.0-1.7]	0.483
Use of lipid-lowering medication, %	165	21	13	0.014
Systolic blood pressure, mm Hg	129	147 ± 21	141 ± 21	0.007
Diastolic blood pressure, mm Hg	129	78 ± 9	76 ± 9	0.064
Use of antihypertensive medication, %	170	69	46	<0.001
Estimated glomerular filtration rate (eGFR), ml/min/1.73m ²	170	77 ± 19	80 ± 16	0.057
Total energy intake, kcal/day	168	1909 ± 645	1967 ± 726	0.367
Ever smoker, %	170	62	60	0.638
Alcohol intake, none/moderate/high ^b	173	24/49/27	16/53/31	0.046
Physical activity score (/1000)	170	3.37 [1.18-6.24]	4.92 [2.94-7.83]	<0.001
Prior cardiovascular disease, %	173	55.5	48.2	0.101
Body composition by DXA				
Total body fat, %	148	37 ± 11	34 ± 9	0.005
Trunk fat mass, kg	148	15.8 ± 13.2	13.2 ± 5.0	<0.001
Trunk lean mass, kg	148	25.0 ± 4.8	24.5 ± 4.7	0.230
Peripheral fat mass, kg	148	13.8 ± 6.0	11.8 ± 4.1	<0.001
Peripheral lean mass, kg	148	19.8 ± 4.5	19.5 ± 4.6	0.533

Data are means ± SD, frequencies (%) or medians [inter-quartile range].

^a n with data differs per variable;^b defined as: 0 glasses/day (none), 1-2 glasses/day in men or 1 glass/day in women (moderate) and >2 in men or >1 glasses/day in women (high).

Chapter 3

Microalbuminuria and cardiovascular autonomic dysfunction are independently associated with cardiovascular mortality: evidence for distinct pathways.

The Hoorn Study.

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Abstract

Objective — Microalbuminuria is associated with cardiovascular mortality, particularly among individuals with type 2 diabetes, but the mechanisms underlying this association are not completely understood. Microalbuminuria is known to be associated with cardiovascular autonomic dysfunction (C-AD), and C-AD in turn is associated with cardiovascular mortality. The purpose of this study, therefore, was to investigate whether C-AD can explain the relationship between microalbuminuria and cardiovascular mortality.

Research Design and Methods— We studied 490 individuals from a population-based cohort of individuals aged 50–75 years who were followed for a median period of 13.6 years. Microalbuminuria was defined as an albumin-to-creatinine ratio ≥ 2.0 mg/mmol in an early-morning spot-urine sample. Ten parameters reflecting different aspects of cardiovascular autonomic function were measured and compiled into a total score of C-AD (mean of separate z-scores). The association between C-AD and microalbuminuria was estimated by multiple linear regression, and relative risks (RRs) for cardiovascular mortality were estimated by Cox proportional hazards analyses.

Results — After adjustments for age, sex, glucose tolerance status, and other risk factors, C-AD was associated with microalbuminuria ($\beta=0.16$ [95% CI 0.01– 0.33]), and both microalbuminuria (RR 2.09 [1.07– 4.08]) and C-AD (1.74 [1.04 –2.89]) were associated with cardiovascular mortality. These associations did not change after further mutual adjustment for C-AD (2.13 [1.09–4.17]) or microalbuminuria (1.76 [1.05–2.94]), respectively.

Conclusions— Both microalbuminuria and C-AD are independently associated with cardiovascular mortality, and the excess mortality attributable to microalbuminuria cannot be explained by C-AD.

Introduction

Microalbuminuria is associated with an increased risk of cardiovascular disease and mortality (1). This association is independent of other known cardiovascular risk factors such as hypertension, dyslipidemia, obesity, smoking, and impaired renal function (1,2). Several mechanisms, notably endothelial dysfunction and low-grade inflammation, have been proposed to explain, at least in part, the increased risk of cardiovascular mortality in individuals with microalbuminuria (3). Cardiovascular autonomic dysfunction (C-AD) could potentially constitute another such mechanism.

Indeed, we as well as others have previously shown that C-AD is associated with microalbuminuria, especially in individuals with impaired glucose metabolism (IGM) and type 2 diabetes (4–6). Two proposed mechanisms explaining this association are, first, a disturbance in glomerular arteriolar autoregulation, which in turn may result in an inability to counteract glomerular hypertension (7), and, second, a reduced drop in nightly blood pressure due to C-AD, both of which may result in microalbuminuria (8). In addition, C-AD is associated with cardiovascular mortality (9,10) and can potentially link microalbuminuria to cardiovascular mortality by arrhythmogenic or atherogenic effects, for example, by promoting vascular calcification and arterial stiffness (11).

In view of these considerations, we investigated, in a prospective cohort study, whether C-AD can explain the relationship between microalbuminuria and cardiovascular mortality or, alternatively, whether both microalbuminuria and C-AD are independently associated with cardiovascular mortality. These hypotheses have never been investigated in the general population and could have clinical relevance because the first hypothesis suggests that C-AD should be targeted to decrease mortality risk in individuals with microalbuminuria and, conversely, the second hypothesis suggests that both microalbuminuria and C-AD can be used for estimating the risk of cardiovascular mortality.

Research Design and Methods

We used data from the Hoorn Study, a population-based cohort study on glucose metabolism and other cardiovascular risk factors in a general Caucasian population, which has been described in detail previously (12). In brief, men and women aged 50–75 years were randomly selected from the population register of the town of Hoorn, the Netherlands; 2,484 subjects participated (response rate 71%). Baseline examinations were conducted from October 1989 until February 1992. All subjects had a 75-g oral glucose

tolerance test, except those in whom type 2 diabetes had previously been diagnosed. An extensive metabolic and cardiovascular investigation was performed in an age-, sex-, and glucose tolerance–stratified, random subsample of 631 participants (89% of those invited), which is used in the present study (12).

Participants without representative urine samples available ($n = 45$) and/or who did not complete at least 7 of the 10 autonomic function tests ($n = 31$), had a history of neurological disease ($n = 5$), and were using ACE inhibitors ($n = 33$) and/or drugs known to influence autonomic nerve function (namely antiparkinson drugs, phenytoin, antihistamines, or parasympatholytic, parasympathomimetic, and sympathomimetic drugs) ($n=49$) were excluded from the analyses. The present study, therefore, consisted of 490 individuals: 305 with normal glucose metabolism, 71 with IGM (including those with impaired fasting glucose and/or impaired glucose tolerance), and 114 with type 2 diabetes.

The Hoorn Study was approved by the ethical review committee of VU University Medical Centre, Amsterdam, the Netherlands. Informed consent was obtained from all participants.

Baseline measurements

Urinary albumin concentration was measured in a first voided sample by rate nephelometry (Array Protein System, Beckman Coulter, Galway, Ireland) with a detection threshold of 6.2 mg/l (intra- and interassay coefficients of variation of 5 and 8%, respectively). Urinary creatinine was measured with a modified Jaffé method. Microalbuminuria was present if the albumin-to-creatinine ratio (ACR) was in the range of 2.0–30 mg/mmol (5). The average value of the ACR was used if two representative samples ($n = 154$) were available. Excluding subjects with an ACR > 30 mg/mmol (i.e., macroalbuminuria, $n = 4$) did not materially affect any of the results, which are therefore presented for the entire group.

Cardiovascular autonomic function tests were performed as described in detail elsewhere (9). In brief, participants were asked to refrain from smoking and drinking coffee for at least 2 h before the assessments. A light meal > 1 h before the measurements was allowed. Tests took place between 8:30 A.M. and 4:00 P.M. in a quiet location with room temperature of 19–22°C after a resting period of at least 10 min. Ten parameters of cardiac autonomic function were derived from the R-R interval and finger systolic blood pressure (SBP) continuous recordings by a computerized data analyses system developed locally by the Department of Medical Physics, VU University, Amsterdam, the Netherlands (13). Tests were performed under three conditions: during spontaneous breathing over 3

min in the supine position (mean of all normal to normal R-R intervals [MeanNN], SD of all normal to normal R-R intervals [SDNN], low-frequency [LF] power, high-frequency [HF] power, and $LF/(LF + HF)$), during six deep breaths over 1 min in the supine position (expiration-inspiration [EI] difference and baroreflex sensitivity [BRS]), and during an active change from lying to standing (SBP difference, R-R interval maximum [RRmax], and R-R interval maximum divided by minimum [RRmax/min]) (for further explanation of measurements, see supplementary **Table A1**, available in an online appendix at <http://care.diabetesjournals.org/cgi/content/full/dc08-1544/DC1>). We thus obtained results in four tests that reflect heart rate or blood pressure changes due to certain “maneuvers,” in this case deep breathing or standing up (i.e., EI difference, RRmax, RRmax/min, and SBP difference) (14), five tests of spectral analyses of heart rate variability (HRV) [i.e., MeanNN, SDNN, LF power, HF power, and $LF/(LF + HF)$] (15), and one BRS measurement (16). In addition, these tests were grouped into those representing predominantly parasympathetic (i.e., EI difference, RRmax, and HF power), sympathetic (i.e., SBP difference), or both functions (i.e., Mean-NN, SDNN, LF power, $LF/(LF + HF)$, BRS, and RRmax/min) (15,16). BMI, waist-to-hip ratio (WHR), SBP and diastolic blood pressure (DBP), levels of fasting plasma glucose; A1C; insulin; total, HDL, and LDL cholesterol; triglycerides; creatinine; and smoking status were measured as described elsewhere (5,9,12). Glomerular filtration rate was estimated by the short Modification of Diet in Renal Disease equation. Hypertension was defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg and/or the use of antihypertensive drugs. Prior cardiovascular disease (CVD) was defined when individuals had (any of the following): a history of myocardial infarction, abnormalities on a resting electrocardiogram (Minnesota codes 1.1–1.3, 4.1–4.3, 5.1–5.3, or 7.1), previous coronary bypass surgery or angioplasty, peripheral arterial bypass, or nontraumatic amputation, or an ankle-brachial index of <0.9 in either leg.

Follow-up

Data on the participants' vital status up to 1 January 2005 were collected from the mortality register of the municipality of Hoorn. Information on cause of death was extracted from the medical records of the general practitioners and the local hospital and coded according to the ICD-9. Cardiovascular mortality, including sudden death, was defined by ICD-9 codes 390–459 and 798. Information on cause of death could not be obtained for 20 of the deceased individuals. All subjects were followed until death or end of follow-up, at which time they were censored.

Statistical analyses

Baseline characteristics between survivors and nonsurvivors were compared with the use of Student's t or χ^2 tests. We computed z scores ([individual's observed value - population

mean]/SD) for each of the 10 measurements of C-AD. All z scores (except SBP difference) were inverted and averaged into a total score ("C-AD total score"), so that higher values reflect greater C-AD. The association between microalbuminuria and C-AD total score was investigated with multiple linear regression analyses. Kaplan-Meier survival curves were plotted for cardiovascular mortality among individuals with normoalbuminuria versus microalbuminuria and similarly across tertiles of the C-AD total score, and differences between groups were tested with a log-rank test. Cox proportional hazards regression models were used to calculate the crude and adjusted relative risks (RRs) and respective 95% CI of microalbuminuria (versus normoalbuminuria) and of the C-AD total score (per SD [= 0.605] increase) for cardiovascular and all-cause mortality. Two-sided $P < 0.05$ was considered statistically significant. All analyses were performed with SPSS (version 15.0).

Results

Subjects excluded from the analyses ($n = 141$) were older, more often had type 2 diabetes, and had a worse risk profile than the subjects included ($n = 490$) (data not shown). Median duration of the follow-up was 13.6 (range 0.52–15.19) years. During the follow-up period, 141 (28.8%) participants died, of whom 53 (37.6%) died of CVD. At baseline, individuals who died had a worse C-AD total score, more often had microalbuminuria and were older, more often were men and had type 2 diabetes and hypertension, and had a higher A1C and WHR as compared with the survivors (**Table 1**).

Association between microalbuminuria and C-AD

C-AD total score was associated with microalbuminuria ($\beta = 0.33$ [95% CI 0.16–0.51]) (**Table 2**, model 1). After adjustment for potential confounding factors, C-AD total score was still borderline associated with microalbuminuria (0.16 [-0.01 to 0.33]) (**Table 2**, model 3).

Comparable results were obtained when tests were combined on the basis of their methodology or on the part of the autonomic nervous system they predominately represent. All individual C-AD tests, except LF/(LF + HF), were positively associated with microalbuminuria (although not all statistically significantly so) (data not shown).

Table 1 - Baseline characteristics of survivors vs. non survivors

Characteristics	Survivors (n=349)	Non-survivors	
		All-cause mortality (n=141)	Cardiovascular mortality (n=53)
Age (years)	62.4 (6.8)	67.1(6.4) [*]	67.7(6.7) [*]
Sex (% men)	43.6	53.9 [*]	50.9
Glucose tolerance status (%)			
NGM	66.5	51.8	43.4
IGM	14.0	15.6	17.0
Type 2 diabetes	19.5	32.6 [*]	39.6 [*]
HbA1c (%)	5.7 (1.1)	6.2 (1.4) [*]	6.4 (1.6) [*]
Systolic blood pressure (mmHg)	136.2 (18.5)	142.8 (19.6) [*]	147.2 (21.6) [*]
Diastolic blood pressure (mmHg)	82.0 (9.3)	83.3 (11.2)	84.4 (10.8)
Use of blood-pressure-lowering drugs (%) ^a	20.9	31.9 [*]	32.1 [*]
Hypertension (%)	46.4	65.2 [*]	69.8 [*]
History of CVD (%)	19.8	27.0	30.2
(Ever) smokers (%)	59.6	71.6 [*]	73.6
WHR	0.90 (0.08)	0.94 (0.08) [*]	0.95 (0.08) [*]
BMI (kg/cm ²)	26.8 (3.7)	27.2 (3.9)	27.4 (3.8)
Serum creatinine (μmol/l)	89.5 (16.1)	95.0(23.0) [*]	97.6 (30.6)
eGFR (ml/min)	69 (11)	66 (13)	64 (15) [*]
Cholesterol (mmol/l)	6.61 (1.13)	6.73(1.31)	6.86 (1.19)
HDL (mmol/l)	1.31 (0.37)	1.26 (0.34)	1.23 (0.31)
LDL (mmol/l)	4.51 (0.99)	4.60 (1.13)	4.77 (1.05)
Use of lipid-lowering drugs (%)	1.7	1.4	1.9
Triglycerides (mmol/l)	1.50 [1.10-2.10]	1.70 [1.20-2.40] [*]	1.80 [1.25-2.40]
EI-difference (ms)	162 [107-237]	131 [83-213] [*]	113 [79-183] [*]
RR _{max} (ms)	254 (96)	217 (88) [*]	223 (91) [*]
RR _{max} /min	1.27 (0.16)	1.19(0.14) [*]	1.21(0.13) [*]
HF-power (ms ²)	183 [85-438]	151[67-363] [*]	145 [77-330]
LF-power (ms ²)	239 [123-525]	160 [68-390] [*]	152 [63-307] [*]
LF/(LF+HF)	0.56 (0.19)	0.52(0.21)	0.50 (0.21) [*]
BRS (ms/mmHg)	9.01 (4.96)	7.91 (4.20) [*]	7.84 (4.14)
SDNN (ms)	36 (16)	33 (19) [*]	32 (19)
Mean NN (ms)	966 (147)	927 (156) [*]	935 (157)
SBP-difference (mmHg)	-6.42 (14.82)	-6.32 (15.17)	-6.45 (16.51)
C-AD total score	-0.08 (0.58)	0.22 (0.61) [*]	0.22 (0.65) [*]
Microalbuminuria (%)	7.7	16.3 [*]	24.5 [*]

Data are presented as frequencies (%), means (±SD) or medians [inter-quartile range (IQR)]

^{*} p <0.05 vs. survivors. NGM: normal glucose metabolism; IGM: impaired glucose metabolism; DM: type 2 diabetes mellitus; CVD; cardiovascular disease; WHR: waist-to-hip ratio; BMI: body mass index; eGFR: estimated glomerular filtration rate; HDL: high density lipoprotein; LDL: low density lipoprotein; C-AD: cardiovascular autonomic dysfunction

For detailed explanation of autonomic function tests and their abbreviations, see Table A1.

^a these include diuretics, alpha-blockers, beta-blockers, calcium channel blockers and other blood pressure lowering drugs but exclude ACE-inhibitors (because subjects using these drugs were excluded from the analyses).

Table 2 - Association between microalbuminuria and C-AD

Dependent variable	Model	β	95% CI	p-value
C-AD total score	1	0.33	0.16; 0.51	<0.001
	2	0.21	0.04; 0.38	0.016
	3	0.16	-0.01; 0.33	0.067
C-AD _{maneuvers}	1	0.34	0.15; 0.53	0.001
	2	0.17	-0.01; 0.35	0.061
	3	0.12	-0.07; 0.30	0.209
C-AD _{BRS}	1	0.40	0.08; 0.71	0.014
	2	0.23	-0.08; 0.54	0.149
	3	0.21	-0.11; 0.53	0.194
C-AD _{HRV}	1	0.31	0.12; 0.51	0.001
	2	0.22	0.03; 0.41	0.025
	3	0.16	-0.04; 0.35	0.110
C-AD _{parasympathetic}	1	0.45	0.22; 0.69	<0.001
	2	0.28	0.05; 0.51	0.017
	3	0.23	-0.01; 0.46	0.055
C-AD _{sympathetic}	1	0.14	-0.18; 0.44	0.394
	2	0.13	-0.19; 0.44	0.433
	3	0.11	-0.22; 0.44	0.503
C-AD _{both}	1	0.32	0.14; 0.49	<0.001
	2	0.23	0.06; 0.41	0.001
	3	0.18	0.01; 0.36	0.041

β , indicates difference in C-AD score between individuals with micro- vs. normoalbuminuria;

Model 1: univariate analysis;

Model 2: adjusted for sex, age and glucose tolerance status;

Model 3: model 2 plus adjustments for hypertension, WHR, eGFR, LDL, HDL, triglycerides, smoking and prior CVD.

Association of microalbuminuria and C-AD total score with cardiovascular and all-cause mortality

Both microalbuminuria (RR 3.49 [95% CI 1.87– 6.53]) and C-AD total score (2.54 [1.60– 4.04]) were associated with cardiovascular mortality (**Table 3**, model 1; Fig. 1). These associations were attenuated after adjustment for age, sex, and glucose tolerance status (GTS) (2.23 [1.16–4.29] and 1.81 [1.11–2.94], respectively; model 2). Further adjustment for other cardiovascular risk factors only slightly further attenuated these RRs (model 3), and when the C-AD total score or microalbuminuria was added to these models, the RR attributable to microalbuminuria remained practically unchanged and both microalbuminuria (2.13 [1.09–4.17]) and C-AD total score (1.76 [1.05–2.94]) were independently associated with cardiovascular mortality (model 4). Both microalbuminuria and C-AD total score were also associated with all-cause mortality (**Table 3**, model 1). However, after adjustment for age, sex, GTS, and other cardiovascular risk factors, microalbuminuria (RR 1.33 [95% CI 0.83–2.13]), in contrast to C-AD total score (1.52 [1.11–2.09]), was not independently associated with all-cause mortality (model 3).

Table 3 - Association of microalbuminuria or C-AD with cardiovascular and all-cause mortality

Main determinant	Model	Cardiovascular mortality	All-cause mortality
		RR [95% CI]	RR [95%CI]
Microalbuminuria	1	3.49 [1.87-6.53]	2.12 [1.36-3.21]
	2	2.23 [1.16-4.29]	1.46 [0.92-2.31]
	3	2.09 [1.07-4.08]	1.32 [0.83-2.12]
	4	2.13 [1.09-4.17]	1.33 [0.83-2.13]
C-AD total score	1	2.54 [1.60-4.04]	2.11 [1.58-2.81]
	2	1.81 [1.11-2.94]	1.62 [1.20-2.20]
	3	1.74 [1.04-2.89]	1.52 [1.11-2.08]
	4	1.76 [1.05-2.94]	1.52 [1.11-2.09]

Model 1: univariate analysis;

Model 2: adjusted for sex, age and glucose tolerance status;

Model 3: model 2 plus adjustments for hypertension, WHR, eGFR, LDL, HDL, triglycerides, smoking and prior CVD;

Model 4: model 3, with additional adjustment for respectively C-AD or microalbuminuria;

RR: relative risk of micro- vs. normoalbuminuria, or per standard deviation (=0.605) increase in C-AD total score.

Additional analyses

The associations described above were also investigated by examining autonomic function tests combined on the basis of their methodology or on the part of the autonomic nervous system they predominantly represent (supplementary **Table A2**, available in an online appendix). These additional analyses showed that overall all different groups of tests were associated with increased mortality risk, that these associations were attenuated when further adjusted for age, sex, GTS, and other risk factors; and that none of the specific group of tests explained the association between microalbuminuria and mortality. The associations reported in **Table 3** did not materially change after additional adjustment for use of lipid- or blood pressure-lowering drugs and the presence of leukocytes in urine (tested by microscopy and scored as positive whenever there were more than five leukocytes per high-power field) or when the dichotomous variable hypertension used in those models was replaced by either SBP or pulse pressure (a marker of arterial stiffness) or when analyses were adjusted for prior coronary heart disease instead of prior CVD (data not shown). Finally, we found no evidence of effect modification by GTS in the association between cardiovascular mortality and C-AD total score or microalbuminuria (P for interaction > 0.4 in both cases).

Figure 1. Kaplan-Meier survival plots for cardiovascular mortality among individuals with normo- versus microalbuminuria (A) and across tertiles of C-AD total score (B).

Conclusions

In this prospective study we found that both microalbuminuria and C-AD (estimated from the mean of 10 standardized tests) were independently associated with cardiovascular mortality in a cohort of elderly, Caucasian subjects with and without diabetes. Therefore, C-AD does not explain why microalbuminuria is related to cardiovascular mortality in either the general population or in diabetic subjects.

Recently, HRV was found not to predict the decline of GFR but was independently associated with cardiovascular mortality in middle-aged type 1 diabetic subjects with overt nephropathy (17). In another study, investigating the independent predictive role of GFR, microalbuminuria, and cardiovascular autonomic neuropathy showed the latter to also be associated with all-cause and cardiovascular mortality in subjects with diabetes (18). These findings are in line with those reported herein. Hitherto, no other prospective studies examining the relation of microalbuminuria and C-AD with cardiovascular mortality have been reported. The independent association of both microalbuminuria and C-AD with cardiovascular mortality reported herein indicates that they are linked to cardiovascular mortality by different biological pathways. Several mechanisms linking C-AD to cardiovascular mortality have been proposed (e.g., QT interval prolongation and silent myocardial ischemia) (19). Microalbuminuria may be linked to CVD by a common pathophysiological process (such as endothelial dysfunction or chronic low-grade inflammation) (3). However, the possibility that microalbuminuria reflects the presence of a set of undiscovered factors that are causally related to CVD remains to be further elucidated. Our study shows that C-AD is an unlikely candidate in this regard.

Microalbuminuria is an established risk indicator for CVD. Furthermore, aggressive treatment of microalbuminuria by inhibiting the renin-angiotensin aldosterone system has been shown to decrease renal and cardiovascular risk in individuals with diabetes (20). There is some evidence that C-AD is also a useful risk indicator for CVD, especially in subjects at high risk (type 2 diabetes, hypertension, or prior CVD) (9,21), and this study reinforces that observation. However, whether the improvement in C-AD, e.g., with exercise training or blood pressure- or lipid-lowering drugs (22–24), leads to a better prognosis is unknown. In our study, additional adjustment for use of β -blockers or lipid-lowering drugs had no effect on the associations reported. Therefore, the clinical relevance of treating C-AD in the general population remains unclear and needs to be further examined.

The strengths of our study are the relatively large, truly population-based and prospective design with a long follow-up period. In addition, we characterized subjects' C-AD as comprehensively as possible by conducting 10 autonomic function tests, which are thought to reflect all aspects of cardiovascular autonomic function. Previous cross-sectional studies evaluating the relationship between microalbuminuria and C-AD used less extensive autonomic function tests (4,6). Because we included all three groups of GTS, we were able to analyze possible effect modification of GTS in the relationship between the main determinants, C-AD and microalbuminuria and cardiovascular mortality (but found none).

There are several limitations to our study. First, each of the test parameters of autonomic function was measured with moderate levels of reproducibility (reliability coefficient 50%) (13). Therefore, the results were combined into a C-AD total score, which enabled us to circumvent, at least partially, this problem. Specifically, the results herein reported with the C-AD total score were more powerful than results obtained on the basis of each individual test (separately), because these results would be more strongly affected by misclassification (likely to be random), causing an underestimation of the strength of the associations. Indeed, the associations between each individual test result with cardiovascular mortality were in accordance, although with lower strength, with those as reported herein with the C-AD total score (data not shown). In addition, this approach had the advantage of circumventing the problem of multiple testing (type I errors) that would have occurred when the associations with each test were analyzed separately. However, because each test may represent different parts of autonomic function, some overlapping but others complementary, our C-AD total score does not allow the distinction of potential different contributions of sympathetic and parasympathetic functions to cardiovascular mortality. Therefore, we examined the autonomic function tests combined on the basis of

their methodology or on the part of the autonomic nervous system they predominantly represent in the additional analyses. The results of these alternative combinations of autonomic function tests were comparable to those of the C-AD total score (supplementary **Table A2**), but we acknowledge that these analyses may not have been specific enough, as most authors agree that a clear distinction between parasympathetic or sympathetic dysfunction cannot be made on the basis of these tests. Altogether, we feel that the advantages (i.e., reduced misclassification and avoidance of type I errors without impairment of etiological validity) of the approach used by us outweigh the disadvantages. Second, the spectral analyses were performed during 3 min. Measurement of HRV during a longer period may be more reliable, although measurements obtained in time periods as short as 2 min correlate highly with 24-h measurements (25). Third, evaluation of subjects' C-AD was conducted at baseline only, and, therefore, we were not able to evaluate deterioration of autonomic function during follow-up. Fourth, for most participants microalbuminuria was defined on an ACR measured in one urine sample only. Because microalbuminuria is quite variable from day to day we cannot fully exclude the possibility that some subjects were misclassified. However, we tried to avoid this problem by collecting urine overnight and expressing albumin excretion as ACR. In addition, analyses in the 154 participants for whom two samples were available yielded essentially similar results (data not shown). Finally, we studied an elderly, Caucasian population, and it is not known whether our results can be generalized to other ethnic groups or to younger individuals.

In summary, we have shown that both microalbuminuria and C-AD are independently associated with cardiovascular mortality in an elderly, Caucasian population of individuals with normal glucose metabolism, IGM, and diabetes. These results suggest that microalbuminuria and C-AD are related to cardiovascular mortality by different biological pathways. Therefore, it may be useful to treat not only microalbuminuria but also C-AD in populations at high risk of cardiovascular mortality.

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No potential conflicts of interest relevant to this article were reported.

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SUPPLEMENTAL TABLES

Table A1 - Overview of the ten measurements of cardiovascular autonomic function

<i>Measurement</i>	<i>Unit</i>	<i>Definition</i>
<u>During spontaneous breathing over 3 min in supine position</u>		
Mean NN	ms	the mean of all normal to normal, i.e. sinus rhythm, R-R intervals
SDNN	ms	the standard deviation of all normal to normal i.e. sinus rhythm, R-R intervals
LF-power	ms ²	Low frequency power, in absolute units: energy in the power spectrum between 0.04 and 0.12 Hz
HF-power	ms ²	High frequency power, in absolute units: energy in the power spectrum between 0.12 and 0.40 Hz
LF/ (LF+HF)		the ratio of LF-power to the sum of LF- and HF-power
<u>During six deep breaths over 1 min in supine position</u>		
EI-difference	ms	the mean expiration-inspiration difference in R-R intervals over six consecutive breaths*
BRS	ms mmHg ⁻¹	A measure of baroreflex sensitivity, computed as gain, i.e. ratio of the energy in the cross-spectrum of systolic blood pressure and R-R intervals, and the energy in the power spectrum of the R-R interval; all between 0.05 and 0.15 Hz and with a squared coherence (γ^2) of 0.5 or higher
<u>During an active change in position from lying to standing</u>		
RRmax	ms	the difference between the mean R-R interval during 1 minute of rest prior to standing up and the minimum R-R interval within 15 seconds after standing up
RRmax/min		maximum R-R interval between 15 and 30 seconds after standing up divided by minimum R-R interval within 15 seconds after standing up
SBP difference	mmHg	systolic blood pressure after standing up (mean of 1.5-2 minutes after standing) minus systolic blood pressure in supine position

*The frequency of breathing at six breaths per minute was dictated by the investigator. When off-line spectral analysis showed that breathing was not performed at the appropriate frequency, the record was discarded. RR-intervals were obtained from an electrocardiogram by a hardware QRS detector with an accuracy of one millisecond. Blood pressure was recorded using the Finapres™ device (Finger Arterial Blood Pressure, Ohmeda BP2000); SBP values were obtained from the 200 Hz sampled continuous blood pressure signal by means of an automatic procedure, which was verified by visual inspection.

Table A2 - Association between microalbuminuria and cardiovascular mortality before and after adjustment for different C-AD tests

Main determinants	Model A	Model B	Model C
	RR [95% CI]	RR [95% CI]	RR [95% CI]
Microalbuminuria	3.47 [1.86-6.49]	2.07 [1.06-4.04]	2.13 [1.09-4.16]
C-AD _{maneuvers} [*]	2.43 [1.59-3.71]	1.53 [0.95-2.47]	1.57 [0.97-2.55]
Microalbuminuria	3.34 [1.64-6.81]	1.95 [0.92-4.16]	1.97 [0.92-4.19]
C-AD _{BRS} [†]	1.27 [0.90-1.80]	1.10 [0.77-1.56]	1.10 [0.77-1.58]
Microalbuminuria	3.77 [1.99-7.13]	2.09 [1.05-4.15]	2.08 [1.04-4.15]
C-AD _{HRV} [‡]	1.96 [1.25-3.08]	1.60 [1.00-2.56]	1.60 [0.99-2.58]
Microalbuminuria	3.49 [1.87-6.53]	2.09 [1.07-4.07]	2.11 [1.08-4.11]
C-AD _{parasympathetic} [§]	1.83 [1.30-2.59]	1.39 [0.95-2.04]	1.40 [0.96-2.04]
Microalbuminuria	3.21 [1.58-6.56]	2.11 [0.99-4.52]	2.10 [0.95-4.48]
C-AD _{sympathetic}	1.02 [0.75-1.38]	1.13 [0.81-1.56]	1.13 [0.81-1.57]
Microalbuminuria	3.49 [1.87-6.53]	2.09 [1.07-4.07]	2.03 [1.04-3.99]
C-AD _{both} [¶]	2.05 [1.29-3.26]	1.63 [1.02-2.63]	1.62 [1.00-2.62]

Model A: univariate analysis (note: the RRs differ across the different sets of analyses due to different number of subjects who completed the specific tests under investigation; specifically, data on individual tests were missing due to incompleteness or incorrect performance of the test or insufficient quality of the data for processing: maneuvers (n=4), BRS (n=60) and HRV (n=34));

Model B: analyses adjusted for age, sex, glucose tolerance status and other risk factors (= Model 3 in Table 3);

Model C: Model B plus mutual adjustment for different C-AD tests and microalbuminuria;

^{*} Variables: EI-diff, RRmax, RRmax/min, SBP-diff (SD=0.66);

[†] Variable: BRS (SD=1.00);

[‡] Variables: MeanNN, SDNN, LF-power, HF-power, LF/(LF+HF) (SD=0.65);

[§] Variables: EI-diff, RRmax, HF-power (SD=0.82);

^{||} Variable: SBP-diff (SD=1.00);

[¶] Variables: MeanNN, SDNN, LF power, LF/(LF+HF), BRS, RRmax/min (SD=0.60);

RR: relative risk of micro- versus normoalbuminuria, or per standard deviation (SD) increase in each different C-AD scores. For explanation of autonomic function tests and their abbreviations, see Table A1

The background of the entire page is a close-up, high-resolution photograph of numerous smooth, light-colored stones, possibly river stones or pebbles. The stones vary in shape, size, and shade, ranging from off-white to light gray. They are densely packed, creating a textured, organic pattern that fills the entire frame. The lighting is soft and even, highlighting the smooth surfaces and subtle variations in the stones' colors.

Chapter 4

The metabolic syndrome is associated with maladaptive carotid remodeling.

The Hoorn Study.

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Abstract

Background - The metabolic syndrome (MetS) is associated with an increased risk of stroke. Arterial remodeling could play an important role herein as maladaptive remodeling is a risk factor for stroke. The purpose of this study was to investigate whether MetS was associated with maladaptive remodeling of the carotid artery and if any such association was independent of hemodynamic variables.

Methods - We studied 385 (n = 195 women) nondiabetic, elderly subjects. A MetS z-score (average of sex-specific z-scores of the five MetS traits) was constructed. Intima-media thickness (IMT) and interadventitial diameter (IAD) were assessed by ultrasonography, and lumen diameter (LD), and circumferential wall stress (CWS) were calculated. Multiple linear regression analysis was used to investigate the association between MetS and carotid remodeling.

Results - After adjustment for age, sex, height, prior cardiovascular disease (CVD), dyslipidemia, and smoking, MetS was independently associated with a greater IAD (regression coefficient (β) per S.D. increase in MetS z-score (95% confidence interval), 0.45 mm (0.28; 0.63)), LD (0.41 mm (0.25; 0.58)) and CWS (5.56 kPa (3.71; 7.42)). These associations were attenuated after additional adjustment for inflammatory, metabolic and particularly hemodynamic variables, but remained statistically significant. No significant association was found between MetS and IMT (0.020 mm (-0.006; 0.046)).

Conclusions - MetS is associated with maladaptive remodeling of the carotid artery, which is the result of changes in LD, IAD, and, to a lesser extent, IMT. This process is independent of hemodynamic variables. Whether this association and process will be observed in a broader population and explains the increased risk of stroke in MetS deserves further study.

Introduction

The incidence of ischemic stroke increases with the increasing prevalence of the metabolic syndrome (MetS), a construct defined by the coexistence of central obesity, dyslipidemia, elevated blood pressure, and impaired glucose metabolism (1–4). The underlying pathophysiology of the MetS–stroke relationship, however, remains poorly understood, although it is likely that changes in both structural and functional arterial properties play an important role.

Arterial remodeling, which is the change of structural arterial properties over time in response to inflammatory, metabolic, and/or hemodynamic alterations within the arterial environment, is thought to be an adaptive mechanism aimed at keeping shear stress within certain limits (5). It is characterized by an increase in intima-media thickness (IMT) and by widening of the interadventitial diameter (IAD). Maladaptive remodeling (i.e., remodeling accompanied by an increase in circumferential wall stress (CWS) and circumferential wall tension (CWT)) may be associated with stroke, as it has been shown that in stroke patients CWT was higher at the affected side (6). In addition to the above, three different patterns of arterial remodeling have been identified: inward, outward, and compensatory remodeling (7) of which inward and outward remodeling have been associated with stable and unstable plaque formation, respectively (7–9).

In view of these considerations, we investigated the association between MetS and carotid remodeling in a population-based cohort. For the present study, we focused on MetS in nondiabetic individuals, because type 2 diabetes is known to be associated with maladaptive carotid remodeling (10). In addition, we explored whether the relationship between MetS and carotid remodeling (if any) was mediated by inflammatory, metabolic, or hemodynamic factors, as estimated by high-sensitivity C-reactive protein (hsCRP), the homeostatic model for the assessment of insulin resistance (HOMAIR) and adiponectin and pulse pressure (PP), and mean arterial pressure (MAP), respectively.

Methods

Study population

For the present study, we used data from the 2,000 Hoorn Study follow-up examination and the Hoorn Screening Study. Details have been described elsewhere (11,12).

Briefly, the Hoorn Study is a population-based cohort study of glucose metabolism and its complications, which started in 1989. A random sample of 50–75-year-old subjects was

taken from the population register of the town of Hoorn (59,000 inhabitants) in the Netherlands. Of the 3,553 subjects invited for the study 2,540 (71.5%) agreed to participate, of whom 56 were excluded from analyses because they were non-Caucasians (13). The original Hoorn Study cohort thus consisted of 2,484 subjects. In 2000–2001, a follow-up examination was carried out among surviving participants who gave their permission to be recontacted. Specifically, we invited all participants who had diabetes, as determined by a 75-g oral glucose tolerance test or by diabetes treatment ($n = 176$) and a random sample of participants who had normal glucose tolerance ($n = 705$) or impaired glucose tolerance ($n = 193$), at the 2nd examination. Of the 1,074 individuals invited, 648 (60.3%) persons participated.

Among the nonparticipants, the main reasons for nonparticipation in the 2000–2001 follow-up examination were lack of interest (30%) or comorbidity (23%). Other reasons were advanced age (7%), unwillingness to travel (6%), participation considered too time-consuming (6%), and miscellaneous reasons (15%), whereas 13% gave no reason (14). Additionally, we invited 217 individuals with type 2 diabetes mellitus from the Hoorn Screening Study, of whom 188 (87%) participated (12). All participants underwent a glucose tolerance test, except those with previously diagnosed diabetes ($n = 67$). Data on 14 individuals were missing because of logistical problems. Glucose tolerance was defined according to the 1999 World Health Organization criteria. Altogether, this follow-up cohort thus consisted of 822 individuals (290 with normal glucose metabolism, 187 with impaired glucose metabolism, and 345 with type 2 diabetes). Because, we focused on MetS in nondiabetic individuals, all those with type 2 diabetes were excluded from the present study. The final eligible study population for present study thus consisted of 477 individuals (15). The Hoorn study was approved by the local Ethical Review Committee and written informed consent was obtained from all participants.

MetS

We calculated a MetS z-score to take into account the continuous nature of the association between each of the MetS traits and cardiovascular disease (CVD). This MetS z-score was calculated as follows: for each of the individual traits of MetS a sex-specific z-score was calculated according to the formula ((individual value – study population mean)/study population S.D.) (15,16) The high-density lipoprotein-cholesterol z-score was inverted to indicate increasing risk with increasing value; the blood pressure z-score was based on the systolic value only, because systolic pressure has the strongest effect on stroke. The resulting five z-scores were then averaged into the overall MetS z-score. In addition, MetS was defined according to the updated National Cholesterol Education Program criteria (17). Accordingly, MetS was diagnosed when three or more of the

following five risk factors were present: (i) systolic blood pressure ≥ 130 mm Hg and/or diastolic blood pressure ≥ 85 mm Hg or current drug therapy for known hypertension; (ii) high-density lipoprotein-cholesterol < 1.03 mmol/l in men and < 1.29 mmol/l in women or current drug treatment for low high-density lipoprotein-cholesterol; (iii) triglycerides ≥ 1.7 mmol/l or current drug treatment for hypertriglyceridemia; (iv) fasting plasma glucose ≥ 5.6 mmol/l; and (v) waist circumference ≥ 102 cm in men and ≥ 88 cm in women. Finally, the participants were also classified according to the number of MetS risk factors present (i.e., 0, 1, 2, or ≥ 3).

Carotid arterial properties

Carotid wall properties were determined by ultrasonography as described in detail previously (11). Briefly, a single observer performed right carotid ultrasonography and measurements were taken at 10-mm proximal from the carotid bulb in the absence of clear carotid plaques, with the use of an ultrasound scanner equipped with a 7.5-MHz linear probe (350 Series; Pie Medical, Maastricht, the Netherlands). The scanner was connected to a PC equipped with vessel wall movement detection software and an acquisition system (Wall Track System; Pie Medical). This setup enables measurements of diastolic IAD (i.e., the difference in position of the anterior and posterior arterial wall markers) and IMT (i.e., the distance from the leading edge interface between lumen and intima to the leading edge interface between media and adventitia) (18,19). The mean IAD and IMT of three consecutive measurements were used in the analyses.

From IAD and IMT, lumen diameter (LD) was calculated as $LD \text{ (mm)} = IAD - (2 \cdot IMT)$. CWT and CWS can be calculated according to Laplace's law; i.e., $P \times r/w$, where P is central transmural pressure, which can be expressed as either "pulsatile" or "mean" pressure, r is the lumen radius of the carotid artery, and w is the carotid wall thickness (here taken as IMT) (20). For the present study, pulsatile and mean CWS and CWT were calculated as follows: pulsatile CWT (kPa) = $PP \text{ (LD/2)}$, where PP is the local carotid pulse pressure estimated by calibration (21) of the distension waveforms (22). Pulsatile CWS was calculated as $CWS \text{ (kPa)} = CWT/IMT$. In addition, mean CWS and CWT were calculated as $CWT \text{ (kPa)} = MAP \text{ (LD/2)}$ and $CWS \text{ (kPa)} = \text{mean CWT}/IMT$ (23,24).

Other measurements

Health status, medical history, medication use, and smoking habits were assessed by questionnaires (13). During the examination of the carotid artery in supine position, brachial artery systolic and diastolic pressures were assessed in the left upper arm at 5-min interval over a 45-min period with an oscillometric device (Press-Mate, BP-8800; Colin Medical Instruments, San Antonio, TX). Brachial MAP was calculated as $(2 \times \text{diastolic pressure} + \text{systolic pressure})/3$ (25). We determined glucose (fasting and postload),

hemoglobin A1c, insulin, serum total, high-density lipoprotein and low-density lipoprotein-cholesterol, triglycerides, hsCRP, adiponectin, height, weight, waist circumference, hip circumference, waist-to-hip ratio, and body mass index (12–14). Insulin resistance was calculated according to the HOMA-IR model (26). Prior CVD was defined when individuals had any of the following: a history of myocardial infarction, transient ischemic attack, or ischemic stroke, abnormalities on a resting electrocardiogram (Minnesota codes 1.1–1.3, 4.1–4.3, 5.1–5.3, or 7.1), undergone coronary bypass surgery or angioplasty, peripheral arterial bypass or nontraumatic amputation and/or ankle-brachial index of <0.9 in either leg.

Statistical analyses

All analyses were conducted with SPSS (version 15.0; Chicago, IL). Linear regression analysis was used to analyze the relationship between the MetS z-score and the individual carotid properties. These analyses were then adjusted for age, sex, and height (model 1) and other potential confounders, i.e., low-density lipoprotein, use of lipid-lowering drugs, prior CVD, and smoking (model 2). Finally, the models were adjusted for inflammatory, metabolic, or hemodynamic potentially explanatory determinants, i.e., hsCRP (model 3), HOMA-IR (model 4A) and adiponectin (model 4B), MAP and PP (model 5A and B), respectively.

All associations are reported as regression coefficients (β s) with their 95% confidence intervals. Assumption of linearity intrinsic to the method of data analyses adopted was checked by inspection of the residuals by predicted values scatter plots (27). After assessment of the main effects, interaction terms were introduced into the models to investigate whether the association between MetS and arterial remodeling differed between men and women or between subjects with normal glucose metabolism or impaired glucose metabolism. A two-sided P value of <0.05 was considered statistically significant, except for the interaction analyses where P values <0.10 were considered statistically significant.

Results

Of the 477 eligible participants, 92 were excluded from the present study, because of qualitatively unsatisfactory ultrasound examinations (mostly attributable to obesity) (n = 28) and/or missing data on key variables (n = 83). The present study sample therefore consisted of 385 participants. The excluded participants were older, more obese, and had a higher blood pressure and fasting glucose level than those included (data not shown).

Clinical characteristics by MetS status

The clinical characteristics of the study population according to the presence or absence of MetS are shown in **Table 1**. The prevalence of MetS (≥ 3 risk factors) was 35.3% ($n = 136$) and did not differ between men (34.7%, $n = 66$) and women (35.9%, $n = 70$).

Participants with MetS had greater IAD, IMT, LD, MAP, and carotid PP compared with participants without MetS (**Table 2**). In addition, participants with MetS had greater CWS and CWT compared with those without MetS. **Figure 1** shows the age-, sex-, and height-adjusted mean values of the carotid properties according to the presence or absence of MetS.

Table 1. Baseline characteristics according to absence or presence of MetS

Characteristic	Metabolic syndrome		p-value
	Absent (n=249)	Present (n=136)	
Age (years)	68 $\pm$ 6	68 $\pm$ 6	0.937
Sex (% male)	49.8	48.5	0.812
<i>Traits of MetS</i>			
Systolic blood pressure (mmHg)	134 $\pm$ 18	145 $\pm$ 17	<0.001
Diastolic blood pressure (mmHg)	74 $\pm$ 9	78 $\pm$ 8	<0.001
HDL cholesterol (mmol/l)	1.56 $\pm$ 0.40	1.33 $\pm$ 0.40	<0.001
Triglycerides (mmol/l)	1.00 [0.85-1.40]	1.80 [1.30-2.20]	<0.001
Fasting glucose (mmol/l)	5.49 $\pm$ 0.48	5.93 $\pm$ 0.46	<0.001
Waist (cm)	88 $\pm$ 9	99 $\pm$ 9	<0.001
<i>Other characteristics</i>			
(Current) smoking (%)	16	17	0.843
Antihypertensive use (%)	17	36	<0.001
Height (cm)	170 $\pm$ 9	170 $\pm$ 9	0.364
BMI (kg/m ²)	25.2 $\pm$ 2.5	28.3 $\pm$ 3.0	<0.001
WHR	0.89 $\pm$ 0.08	0.95 $\pm$ 0.83	<0.001
Total cholesterol (mmol/l)	5.8 $\pm$ 1.0	6.0 $\pm$ 1.0	0.150
LDL cholesterol (mmol/l)	3.75 $\pm$ 0.92	3.78 $\pm$ 0.88	0.700
Treatment with lipid-lowering drugs (%)	11	16	0.137
Post-load glucose (mmol/l)	6.05 $\pm$ 1.58	7.12 $\pm$ 1.73	<0.001
HbA1c (%)	5.7 $\pm$ 0.4	5.8 $\pm$ 0.4	<0.001
Fasting insulin (pmol/l)	45 [34-57]	68 [50-92]	<0.001
HOMA-IR	1.66 $\pm$ 0.81	2.77 $\pm$ 1.31	<0.001
Prior CVD (%)	43.0	50.7	0.144
Microalbuminuria (%)	9.2	15.4	0.067
Heart rate (bpm)	61 $\pm$ 8	64 $\pm$ 9	0.001
HsCRP (mg/l)	1.40 [0.86-2.65]	2.17 [1.16-5.10]	<0.001
Adiponectin (mg/l)	12.86 $\pm$ 5.92	10.09 $\pm$ 4.59	<0.001

Data are presented as mean $\pm$ SD or median [inter-quartile range (IQR)]

MetS: metabolic syndrome; HDL: high-density lipoprotein; MAP: mean arterial pressure; BMI: body mass index; WHR: waist-to-hip ratio; LDL: low-density lipoprotein; HOMA-IR: Homeostasis Model Assessment for Insulin Resistance; CVD: cardiovascular disease; hsCRP: high-sensitivity C-reactive protein

MetS and lumen and IAD

A greater MetS z-score was associated with greater LD. This association was attenuated after adjustment for age, sex, and height (**Table 3**, model 1) and other potential confounders (i.e., low-density lipoprotein-cholesterol, use of lipid-lowering drugs, smoking, and prior CVD) (**Table 3**, model 2), but remained statistically significant. Additional adjustment for low-grade inflammation (i.e., hsCRP) (**Table 3**, model 3) or metabolic factors (i.e., HOMA-IR and adiponectin) (**Table 3**, models 4A and B) did not importantly change this association. However, the association was partially attenuated by adjustment for hemodynamic factors (i.e., MAP and PP) (**Table 3**, models 5A–B) but remained statistically significant. Similar results were found for the association between the MetS z-score and IAD (**Table 3**).

Table 2. Carotid arterial properties according to the absence or presence of the metabolic syndrome

Arterial property	Metabolic syndrome		p-value
	Absent (n=249)	Present (n=136)	
Interadventitial diameter (mm)	7.63 ± 1.08	8.17 ± 1.18	<0.001
Intima-media thickness (mm)	0.83 ± 0.16	0.85 ± 0.16	0.132
Lumen diameter (mm)	5.97 ± 0.98	6.46 ± 1.12	<0.001
Mean arterial pressure (mmHg)	94 ± 11	101 ± 9	<0.001
Carotid pulse pressure (mmHg)	58 ± 15	64 ± 17	0.001
Pulsatile circumferential wall tension (kPa)	23 ± 8	27 ± 9	<0.001
Pulsatile circumferential wall stress (kPa)	29 ± 9	33 ± 12	<0.001
Mean circumferential wall tension (kPa)	38 ± 8	44 ± 10	<0.001
Mean circumferential wall stress (kPa)	47 ± 12	53 ± 16	<0.001

Results are expressed as means ± SD

MetS and IMT

A greater MetS z-score was associated with greater IMT. This association lost, however, statistical significance after adjustment for age, sex, and height (**Table 3**, model 1). MetS and circumferential wall tension and stress A greater MetS z-score was associated with greater pulsatile CWT and CWS. These associations were attenuated after adjustment for age, sex, and height (**Table 3**, model 1) and other potential confounders (**Table 3**, model 2), but remained statistically significant. After additional adjustment for low-grade inflammation (**Table 3**, model 3), metabolic (**Table 3**, models 4A and B), or hemodynamic factors (**Table 3**, model 5A), the associations between the MetS z-score and CWT and CWS

remained statistically significant. When the analyses were repeated with the mean CWS and CWT (by replacing local PP in their formulas by MAP), similar results were obtained (**Tables 4 vs. 3**).

Additional analyses

Further adjustment for heart rate, (micro-)albuminuria, use of angiotensin-converting enzyme-inhibitors or angiotensin II receptor blockers did not materially alter any of the results (data not shown). Additionally, the results did not differ between men and women (P values for interaction all ≥ 0.250) or between those with normal glucose metabolism and impaired glucose metabolism (P values for interaction all ≥ 0.099). With regard to glucose tolerance status, a significant interaction term was found only for CWS (P value for interaction 0.099, all other P values for interaction were ≥ 0.315). Stratified analyses then showed that in those with normal glucose metabolism the association between the MetS z-score and CWS was stronger as compared to those with impaired glucose metabolism (β and 95% confidence interval: 8.03 kPa (5.34; 10.73)) vs. 4.89 kPa (2.09; 7.69), respectively).

When the analyses were repeated by replacing the MetS z-score by the z-scores of the individual traits of MetS, analyses showed that blood pressure and waist circumference were the strongest determinants of carotid remodeling (**Table 5**). Finally, the results were not materially altered if the analyses were conducted with MetS expressed as a dichotomized variable (**Tables 6 and 7**) or according to the number of MetS traits present (data not shown).

Figure 1. Age-, sex-, and height-adjusted mean values of LD, IAD, IMT, CWT and CWS according to the presence or absence of MetS. * $p < 0.05$.

CWS, circumferential wall stress; CWT, circumferential wall tension; IAD, interadventitial diameter; IMT, intima-media thickness; LD, lumen diameter; MetS, metabolic syndrome

Table 3. Carotid remodeling (pulsatile CWS and CWT) according to the MetS z-score

Model	LD (mm)		IAD (mm)		IMT (mm)		CWT (kPa)		CWS (kPa)	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
1	0.44 ^c	0.27; 0.60	0.48 ^c	0.31; 0.65	0.020	-0.006; 0.046	4.99 ^c	3.67; 6.32	5.71 ^c	3.87; 7.54
2	0.41 ^c	0.25; 0.58	0.45 ^c	0.28; 0.63	0.020	-0.006; 0.046	4.87 ^c	3.53; 6.20	5.56 ^c	3.71; 7.42
3	0.38 ^c	0.20; 0.55	0.42 ^c	0.23; 0.60	0.018	-0.010; 0.046	4.66 ^c	3.25; 6.07	5.36 ^c	3.40; 7.33
4A	0.63 ^c	0.43; 0.83	0.68 ^c	0.48; 0.89	0.028	-0.004; 0.060	6.99 ^c	5.41; 8.57	8.05 ^c	5.84; 10.26
4B	0.45 ^c	0.28; 0.62	0.48 ^c	0.30; 0.66	0.014	-0.014; 0.042	5.43 ^c	4.04; 6.82	6.48 ^c	4.56; 8.42
5A	0.27 ^b	0.09; 0.45	0.29 ^b	0.10; 0.48	0.012	-0.017; 0.042	1.90 ^b	0.56; 3.23	2.28 ^a	0.34; 4.23
5B	0.30 ^c	0.13; 0.48	0.32 ^c	0.14; 0.50	0.008	-0.020; 0.036	-	-	-	-

Table 4. Carotid remodeling (mean CWS and CWT) according to the MetS z-score

Model	CWT (kPa)		CWS (kPa)	
	β	95% CI	β	95% CI
1	6.35 ^c	4.95; 7.52	7.15 ^c	4.74; 9.56
2	6.21 ^c	4.80; 7.61	6.99 ^c	4.56; 9.42
3	5.88 ^c	4.40; 7.36	6.59 ^c	4.00; 9.17
4A	8.78 ^c	7.14; 10.42	9.85 ^c	6.95; 12.75
4B	7.08 ^c	5.63; 8.53	8.54 ^c	6.03; 11.04
5A	-	-	-	-
5B	4.39 ^c	3.02; 5.75	5.45 ^c	2.93; 7.97

Results are expressed as regression coefficients (β) and respective 95% CI. The regression coefficient β indicates the difference in arterial property (in units) per SD (=0.57) increase in the MetS z-score. ^ap <0.05, ^bp<0.01, ^cp<0.001;

pulsatile CWT=local PP (LD/2), pulsatile CWS= pulsatile CWT / IMT

mean CWT= MAP (LD/2), mean CWS= mean CWT / IMT

Model 1: adjusted for age, sex, height;

Model 2: adjusted for 1 + LDL-cholesterol, use of lipid-lowering drugs, prior CVD, smoking;

Model 3: adjusted for 2 + ln-hsCRP;

Model 4A: adjusted for 2 + ln-HOMA-IR;

Model 4B: adjusted for 2 + adiponectin;

Model 5A: adjusted for 2 + MAP;

Model 5B: adjusted for 2 + local PP

Table 5. Carotid arterial remodeling according to each MetS trait separately

<i>MetS trait</i>	LD (mm)		IAD (mm)		IMT (mm)		Pulsatile CWT (kPa)		Pulsatile CWS (kPa)	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
Blood pressure*	0.31 ^c	0.21; 0.40	0.35 ^c	0.25; 0.45	0.021 ^b	0.006; 0.037	5.66 ^c	5.04; 6.28	6.23 ^c	5.28; 7.19
Waist-circumference	0.20 ^c	0.11; 0.30	0.21 ^c	0.11; 0.32	0.006	-0.009; 0.021	1.63 ^c	0.82; 2.43	1.78 ^b	0.68; 2.89
Glucose tolerance	0.07	-0.02; 0.17	0.10	0.002; 0.20	0.015	0.001; 0.030	0.67	-0.13; 1.47	0.18	-0.90; 1.27
Triglycerides	0.11	0.01; 0.21	0.10	-0.003; 0.20	-0.006	-0.021; 0.009	0.43	-0.38; 1.23	1.13 ^a	0.04; 2.22
HDL†	0.05	-0.05; 0.15	0.05	-0.05; 0.15	-0.001	-0.016; 0.014	0.23	-0.57; 1.04	0.49	-0.60; 1.58

^ap <0.05, ^bp<0.01, ^cp<0.001; Regression coefficient (β) indicates the difference in carotid artery property (in units) per SD increase in the z-score of respective MetS trait.

All models were adjusted for sex, age and height;

* The blood pressure z-score reflects the systolic blood pressure z-score;

† This z-score reflects the inverted HDL z-score;

MetS: metabolic syndrome; LD: lumen diameter; IAD: interadventitial diameter; IMT: intima-media thickness; CWT: circumferential wall tension; CWS: circumferential wall stress; HDL: high-density lipoprotein

Table 6. Carotid remodeling (pulsatile CWS and CWT) according to the presence or absence of MetS

Model	LD (mm)		IAD (mm)		IMT (mm)		CWT (kPa)		CWS (kPa)	
	β	95% CI	β	95% CI	β	95% CI	β	95% CI	β	95% CI
1	0.48 ^c	0.28; 0.67	0.53 ^c	0.33; 0.74	0.027	-0.004; 0.058	4.12 ^c	2.49; 5.74	4.40 ^c	2.16; 6.63
2	0.45 ^c	0.25; 0.65	0.50 ^c	0.30; 0.70	0.025	-0.006; 0.057	3.89 ^c	2.26; 5.51	4.16 ^c	1.92; 6.40
3	0.41 ^c	0.21; 0.61	0.46 ^c	0.25; 0.67	0.023	-0.008; 0.055	3.50 ^c	1.84; 5.16	3.73 ^b	1.44; 6.02
4A	0.60 ^c	0.38; 0.82	0.66 ^c	0.43; 0.89	0.032	-0.004; 0.067	4.85 ^c	3.02; 6.69	5.23 ^c	2.70; 7.77
4B	0.48 ^c	0.28; 0.69	0.52 ^c	0.31; 0.73	0.019	-0.013; 0.052	4.87 ^c	2.55; 7.20	4.27 ^c	2.58; 5.96
5A	0.32 ^b	0.12; 0.52	0.36 ^b	0.15; 0.57	0.019	-0.014; 0.052	1.23	-0.93; 3.39	1.28	-0.21; 2.76
5B	0.37 ^c	0.18; 0.57	0.41 ^c	0.21; 0.61	0.018	-0.014; 0.049	-	-	-	-

Results are expressed as regression coefficients (β) and respective 95% CI. The regression coefficient β indicates the difference in arterial property (in units) between individuals with vs. without MetS. ^ap <0.05, ^bp<0.01, ^cp<0.001.

Pulsatile CWT = local PP (LD/2), pulsatile CWS = pulsatile CWT/IMT.

Model 1, adjusted for age, sex, height; Model 2, adjusted for 1 + LDL-cholesterol, use of lipid-lowering drugs, prior CVD, smoking; Model 3, adjusted for 2 + ln-hsCRP; Model 4A, adjusted for 2 + ln-HOMAIR; Model 4B, adjusted for 2 + adiponectin; Model 5A, adjusted for 2 + MAP; Model 5B, adjusted for 2 + local PP.

CVD, cardiovascular disease; CWS, circumferential wall stress; CWT, circumferential wall tension; HOMAIR, homeostasis model assessment for insulin resistance; hsCRP, high-sensitivity C-reactive protein; IAD, interadventitial diameter; IMT, intima-media thickness; LD, lumen diameter; LDL, low-density lipoprotein; MAP, mean arterial pressure; MetS, metabolic syndrome; PP, pulse pressure.

Table 7. Carotid remodeling (Mean CWS and CWT) according to the presence or absence of MetS

Model	CWT (kPa)		CWS (kPa)	
	β	95% CI	β	95% CI
1	6.07 ^c	4.35; 7.78	6.16 ^c	3.24; 9.08
2	5.85 ^c	4.14; 7.55	5.97 ^c	3.06; 8.88
3	5.35 ^c	3.62; 7.09	5.38 ^c	2.40; 8.35
4A	7.22 ^c	5.31; 9.14	7.31 ^c	4.01; 10.60
4B	6.59 ^c	4.83; 8.35	7.34 ^c	4.34; 10.34
5A	-	-	-	-
5B	4.50 ^c	2.93; 6.06	4.74 ^c	1.86; 7.62

Results are expressed as regression coefficients (β) and respective 95% CI. The regression coefficient β indicates the difference in arterial property (in units) between individuals with vs. without MetS. ^ap <0.05, ^bp<0.01, ^cp<0.001;

pulsatile CWT=local PP (LD/2), pulsatile CWS= pulsatile CWT / IMT

mean CWT= MAP (LD/2), mean CWS= mean CWT / IMT

Model 1: adjusted for age, sex, height;

Model 2: adjusted for 1 + LDL-cholesterol, use of lipid-lowering drugs, prior CVD, smoking;

Model 3: adjusted for 2 + ln-hsCRP;

Model 4A: adjusted for 2 + ln-HOMA-IR;

Model 4B: adjusted for 2 + adiponectin;

Model 5A: adjusted for 2 + MAP;

Model 5B: adjusted for 2 + local PP

MetS: metabolic syndrome; LD: lumen diameter; IAD: interadventitial diameter; IMT: intima-media thickness; CWT: circumferential wall tension; CWS: circumferential wall stress; LDL: low-density lipoprotein; CVD: cardiovascular disease; hsCRP: high-sensitivity C-reactive protein; HOMA-IR: Homeostasis Model Assessment for Insulin Resistance; MAP: mean arterial pressure; PP: pulse pressure

Discussion

The present study on the association between MetS in nondiabetic individuals and carotid remodeling had four main findings. First, MetS was associated with a greater LD, IAD, and IMT, and significantly so for LD and IAD. Second, this constellation of findings identified a pattern of outward remodeling which was accompanied by greater CWS and CWT. The latter particularly suggests that the process of carotid remodeling in MetS is maladaptive. Third, the relationship between MetS and carotid remodeling was not explained by variables closely associated with waist circumference, i.e., hsCRP, adiponectin, or insulin resistance. In addition, hemodynamic factors only partially explained the MetS–carotid remodeling association. Taken both these observations into account, the present results suggest that the remaining factors constituting MetS (i.e., impaired glucose metabolism and dyslipidemia) may also influence the process of arterial remodeling. Finally, the association between MetS and carotid remodeling was similar in men and women. Whether this association explains the increased risk of ischemic stroke in MetS warrants further study.

The results of the present study—in nondiabetic individuals with MetS—are consistent with our earlier findings, which showed that type 2 diabetes was also associated with maladaptive carotid remodeling (10). Taken together, these findings thus support the “ticking clock” hypothesis, which suggests that the elevated CVD risk already starts well before the onset of type2 diabetes (27). This was further emphasized by the fact that, in the present study, unfavorable changes were seen in all carotid properties with an increasing number of MetS risk factors. In addition, the present results were independent of the presence of albuminuria, which extends our previous results on the association between renal function and carotid remodeling (24,28).

The individual MetS component that contributed most to the maladaptive remodeling was blood pressure. This is in line with a previous report in this journal (29) and might be explained by its steady and pulsatile stress upon the arterial wall which causes fracturing of the (pressure-load-bearing) elastin and collagen network (30). We have shown herein that MetS was independently associated with both pulsatile and mean CWS and CWT, even after adjustment for, respectively, the steady (i.e., MAP) and pulsatile (i.e., PP) stress exerted by blood pressure. Our findings thereby extend the Glagov hypothesis (5) which originally stated that the process of arterial remodeling was mediated by shear stress only. This extension of the Glagov hypothesis has also been suggested by others (23,24).

In addition to the above, MetS remained independently associated with maladaptive remodeling even after adjustment for hemodynamic factors, which suggests that the metabolic alterations in MetS (i.e., the combination of dyslipidemia, central fat distribution, and impaired glucose tolerance) play an additive role in the remodeling process in MetS. In this line, a Japanese study showed that dyslipidemia and mean CWT were positively associated in elderly individuals (31). The deleterious contribution of waist circumference, i.e., central obesity, may be explained by its association with adipocyte-derived proinflammatory molecules, known to be involved in (maladaptive) arterial remodeling (32,33). In our study, low-grade inflammation as estimated by hsCRP did not explain this association. This suggests that other adipocyte-derived factors may play an important role. In this line, it has recently been shown that hypoadiponectinemia (which is related to central obesity) in experimental (34) and human (35) studies is associated with ischemic stroke. In the present study, subjects with MetS indeed had lower adiponectin levels than those without MetS, but the association between MetS and carotid remodeling was not explained by hypoadiponectinemia. The potential role of hypoadiponectinemia in stroke therefore warrants further study. Nevertheless, our results suggest that factors related to central obesity other than low-grade inflammation and hypoadiponectinemia may play a role.

Two previous studies investigated carotid remodeling in MetS (36,37). Both studies identified a pattern of outward carotid remodeling. The extent to which the pattern of remodeling in these studies was associated with normalized or increased CWT and CWS was not reported. Consequently, it is difficult to determine whether the changes reflected an adaptive or maladaptive process. Our study expands these previous data by showing that the arterial remodeling response in MetS is maladaptive (with increased CWS and CWT) and independent of inflammatory and hemodynamic factors as expressed by hsCRP and PP and MAP, respectively. The present results support the hypothesis that maladaptive remodeling constitutes one of the mechanisms that explain the MetS–stroke relationship.

We used MetS expressed as a continuous variable to take into account the continuous nature between the MetS traits and CVD. This approach was more robust and avoided the loss of information that would occur with the use of dichotomized variables, and may therefore be more suitable for etiological research as reported herein (15,38,39). Nevertheless, results were similar when the associations with carotid properties were investigated with MetS defined as present/absent instead of the MetS z-score. The present study supports the view that not all MetS traits carry the same burden with respect to cardiovascular risk (39) as in our study blood pressure was the strongest determinant of the MetS–remodeling relationship. However, we have shown that the other MetS components together exert an additive, independent (metabolic) effect on the remodeling process.

The present study had some limitations. First, due to its crosssectional design, any inferences about causality should be made with caution and further prospective studies are warranted. Second, no three-dimensional images of the carotid artery were obtained and therefore our assumptions regarding the geometry of the carotid artery may have caused us to underestimate the MetS–carotid remodeling relationship. In addition, ultrasound cannot discriminate between the intimal and medial layers of the artery. Third, it should be noted that the individuals excluded from the study were older, more obese, and had a higher blood pressure (i.e., had a worse risk profile). Therefore, the reported associations, if anything, may have been slightly underestimated. Finally, we studied a Caucasian, elderly population, and it remains to be established whether the results can be generalized to other ethnicities or to younger individuals.

In conclusion, we have shown that MetS is associated with greater LD and IAD of the common carotid artery in nondiabetic subjects. This outward remodeling process is maladaptive, as shown by an increase in CWT and CWS. This association was independent

of low-grade inflammation and hemodynamic variables and therefore MetS exerts an additive metabolic effect on the remodeling process. Whether this association and process will be observed in a broader population and explains the increased risk of stroke in MetS deserves further study.

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Disclosure

The authors declared no conflict of interest.

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Chapter 5

Clustering of metabolic syndrome traits is associated with maladaptive carotid remodeling and stiffening. A 6-year longitudinal study

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Abstract

Maladaptive arterial remodeling may constitute a mechanism underlying the risk of stroke in individuals with the metabolic syndrome (MetS), but evidence supporting this contention derives from cross-sectional studies only. We, therefore, investigated, in apparently healthy adults, whether changes in MetS status between the ages of 36 and 42 years (never [n=207, reference group], incident [n=31], recovery [n=23], and persistent [n=32]) were associated with changes in carotid interadventitial diameter, lumen diameter, intima-media thickness, circumferential wall tension and stress, and Young's elastic modulus. All data analyses were adjusted for sex, height, and (changes in) age, lifestyle variables, low-density lipoprotein cholesterol, and use of antihypertensive medication. At baseline and as compared with the reference group, individuals with persistent MetS had significantly higher interadventitial diameter, circumferential wall tension, circumferential wall stress, and Young's elastic modulus but not intima-media thickness. In the course of follow-up, these individuals (versus reference group) displayed significantly steeper increases in intima-media thickness (0.011 versus 0.005 mm/y), which were accompanied by significantly steeper increases in interadventitial diameter (0.077 versus 0.032 mm/y) and lumen diameter (0.055 versus 0.023 mm/y) but not circumferential wall stress, which decreased (-0.34 versus 0.12 kPa/y). These findings suggest that increases in intima-media thickness in young adults with the MetS may primarily reflect an adaptive mechanism that attempts to restore local hemodynamic conditions to an equilibrium rather than atherosclerosis, per se. However, carotid adaptations did not restore circumferential wall stress to levels comparable with those of the reference group, and, therefore, outward remodeling was maladaptive. Importantly, individuals who recovered from the MetS restored carotid properties to levels comparable to the reference group, emphasizing the potential for reversibility.

Introduction

Individuals with the metabolic syndrome (MetS), that is, clustering of elevated blood pressure (BP), central obesity, dyslipidemia, and impaired glucose metabolism, have an increase risk of incident cardiovascular disease, in particular, stroke (1). However, the underlying pathophysiology of the MetS-stroke relationship remains poorly understood.

Numerous studies have linked the MetS to increased carotid intima-media thickness (IMT) (2) and or stiffness (3), and both higher levels of carotid IMT and stiffness predict incident cardiovascular disease, in particular, stroke (4,5). Maladaptive arterial remodeling may also constitute a mechanism linking the MetS to increased risk of stroke. Arterial remodeling, that is, the change of structural arterial properties over time in response to hemodynamic and/or metabolic factors operating within the arterial environment, is thought to be an adaptive mechanism aiming at keeping shear stress within certain limits (6). It is characterized by widening of the interadventitial diameter (IAD) and increases in IMT. This process is considered to be maladaptive when, despite remodeling, circumferential wall tension (CWT) and stress (CWS; also designated as tensile stress) remain elevated. Maladaptive arterial remodeling has been linked to stroke by the observation of higher carotid CWT at the affected side of stroke patients (7). Evidence thus far suggests that the MetS is associated with maladaptive arterial remodeling, but this is limited because it derives from cross-sectional studies only (8–12) not all of which reported on CWT and/or CWS together with IAD, lumen diameter (LD), and IMT (10–12).

As snap-shots, cross-sectional studies do not enable a full appreciation of dynamic processes, such as that of arterial remodeling. For example, increases in IMT could precede and lead to subsequent increases in IAD and/or LD, or, alternatively, increases in IMT could result from increased IAD. A longitudinal study is, thus, necessary for a better understanding of the temporal relationships between carotid IMT and diameter. In addition, the appreciation of accompanying (changes in) levels of CWT and CWS can elucidate the extent to which any such observed pattern of arterial remodeling is maladaptive. In view of these considerations, we have examined changes in carotid artery structural and functional properties and local hemodynamic forces as a function of changes (in clustering of traits of) MetS status, in a 6-year longitudinal study among apparently health adults.

Methods

Study Design and Population

Data were derived from the Amsterdam Growth and Health Longitudinal Study, an ongoing observational longitudinal study that started in 1976 with a total inclusion of 698 boys and girls (mean age, 13.1 ± 0.8 years), as described in detail elsewhere (13). In the year 2000, when subjects' mean age was 36.6 years (± 0.6 years), measurements of large artery properties by means of non-invasive ultrasound were included for the first time in this cohort and obtained in 373 subjects (baseline data of the present study) (14–17). In 2006, follow-ups of arterial measurements were obtained in 297 of these individuals, 293 of whom had complete data on carotid artery properties and traits of the MetS at both time points (the sample of the present study). However, baseline characteristics from the 80 individuals without complete follow-up data did not differ from those included (data not shown). Throughout the longitudinal period, anthropometric (body height, weight, and waist circumferences), biological (blood lipids and BP), and lifestyle (smoking status, alcohol consumption, and physical activity levels) variables were assessed according to standard procedures, as detailed elsewhere (14–17). The medical ethical committee of the VU University Medical Center in Amsterdam approved the study, and all of the participants gave written informed consent.

Carotid Artery Properties and BP

At baseline and at follow-up, carotid properties were obtained by means of the same protocol and ultrasound equipment, as described in detail previously (please see online-only Data Supplement) (14–17).

LD was calculated as $IAD - (2 \cdot IMT)$ and the arterial wall cross-sectional (CSA_{IMT}) as $\pi \cdot IMT \cdot (IMT + LD)$; CWT and CWS were estimated according to Laplace's law as $MAP \cdot (LD/2)$ and CWT/IMT , respectively, where MAP is mean arterial pressure (8,9,18,19). The Young's elastic modulus (YEM) was estimated as $IAD/(IMT \cdot DC)$, where DC indicates the distensibility coefficient, which was calculated from IAD, distension, and local pulse pressure (14–17).

Throughout the entire period of ultrasound imaging, systolic (SBP), diastolic (DBP), and MAP were assessed in the left arm at 5-minute intervals with oscillometric devices. Brachial pulse pressure was defined as $SBP - DBP$, and pulse pressure at the level of the carotid artery was calculated by calibration of the diameter-distension waveforms obtained at this and the brachial arteries, as described by Van Bortel et al (20).

Definition of the MetS and Changes in MetS Status Over Time

At follow-up, subject levels of BP were obtained during the arterial assessments only, that is, while subjects were in supine position. We, therefore, deemed the use of the SBP/DBP cutoff values proposed by established definitions of the MetS (eg, National Cholesterol Education Program/International Diabetes Federation) inappropriate, because these are based on readings in the sitting position, which are higher. In addition, levels of fasting plasma glucose were not available at baseline, and, therefore, we used glycated haemoglobin (HbA1c) as a marker of glucose metabolism instead. The presence of MetS traits was defined on the basis of the highest sex-specific quartiles of SBP or DBP, waist circumference, triglycerides, and HbA1c and the lowest sex-specific quartile of high-density lipoprotein cholesterol, as measured at baseline. The cutoff values thereby derived are presented in **Table S2** (available in the online-only Data Supplement) and were used to classify presence or absence of MetS traits at follow-up. Subjects were then assigned to 4 categories according to the number of traits present, that is, 0, 1, 2, and ≥ 3 , and the latter category was used to identify individuals' clustering of traits indicative of the MetS (hereafter referred as "with MetS").

Subjects were classified further into the following 4 groups of changes in MetS status, including never (no MetS at baseline and follow-up; $n=207$), incident (MetS at follow-up but not at baseline; $n=31$), recovery (MetS at baseline but not at follow-up; $n=23$), and persistent (MetS at both baseline and follow-up; $n=32$).

Statistical Analysis

We first examined the validity of the classification of participants' MetS status as adopted herein by testing whether the observed clustering of ≥ 3 traits occurred at levels above those that could be dictated by chance alone (21). This was confirmed by an observed prevalence of the MetS at baseline that was twice higher than which could be expected by chance alone (**Figure S1**).

Generalized estimating equations, which is a statistical method appropriate for analyses of repeated and, thus, correlated data (22), were used to test differences in carotid artery properties, at baseline and at follow-up, and their changes over time between the 4 groups of changes in MetS status. Groups were included in these analyses as a dummy variable and differences tested relative to the reference group (never); differences in changes, also relative to the reference group, were tested by means of group $\cdot$ time interaction terms (model specifications given in the online-only Data Supplement). All of the analyses were adjusted for sex, height (as time-independent covariates), and age, smoking, alcohol consumption, physical activity, low-density lipoprotein cholesterol, and use of antihypertensive medication (as time-varying covariates) (22).

All data are reported in men and women combined, because we did not find any consistent significant interactions with sex. Triglycerides levels, which were positively skewed, were loge transformed before all data analyses. Statistical significance was set at $P < 0.05$ and all analyses were carried out with the use of the STATA software package version 11.2 (STATA Corp, College Station, TX).

Results

General characteristics of the study population, at baseline and follow-up, are shown in **Table 1**, stratified by categories of change in MetS status.

Carotid Properties and Their Changes by Categories of Change in MetS Status

At baseline, individuals with persistent MetS had significantly higher levels of IAD, LD, CWT, CWS, and YEM, but not of IMT, than those who never developed the MetS (**Table 2** and **Figure**). In the course of follow-up, the persistent MetS group displayed significantly steeper increases in IMT (0.011 versus 0.005 mm/y), CSAIMT (0.38 versus 0.14 mm²/y), IAD (0.077 versus 0.032 mm/y), and LD (0.055 versus 0.023 mm/y), whereas changes in CWT (0.33 versus 0.27 kPa/y), CWS (-0.34 versus 0.12 kPa/y), and YEM (0.010 versus 0.002 $10^3 \cdot \text{kPa/y}$) did not significantly differ from those observed in the reference group. Still, at follow-up CWT, CWT, CWS, and YEM remained significantly higher in the group with persistent MetS versus those who never developed the MetS. Individuals who recovered from the MetS had, at baseline, higher levels of CWT and CWS than the group who never developed the MetS but displayed favorable changes in all of the carotid artery properties such that, at follow-up, none of these differed significantly from the group who never developed the MetS. On the other hand, individuals with incident MetS displayed significantly steeper increases in CWT (0.56 versus 0.27 kPa/y) and also more adverse patterns of change in the other carotid properties (albeit not significantly different), such that, at follow-up, these individuals had significantly higher IAD, CWT, and YEM than those who never developed the MetS.

Table 1. Characteristics of the study population at baseline and at follow-up by categories of changes in metabolic syndrome status

Characteristics	Time	Metabolic Syndrome status			
		Never (n=207)	Incident (n=31)	Recovery (n=23)	Persistent (n=32)
Male sex, %		44.9	54.8	52.2	50.0
Age, years	Baseline	36.5±0.6	36.3±0.7	36.8±0.5	36.7±0.6
	Follow-up	42.6±0.6	42.4±0.7	42.8±0.6	42.7±0.6
Traits of the Metabolic Syndrome					
Systolic blood pressure, mmHg	Baseline	113.6±10.3	120.2±10.7	121.3±8.7	126.9±14.6
	Follow-up	113.5±10.1	124.5±15.3	117.6±11.2	125.0±17.0
Diastolic blood pressure, mmHg	Baseline	63.1±6.5	67.6±6.9	69.2±6.2	71.1±7.5
	Follow-up	68.7±7.8	75.7±7.9	71.2±6.1	75.1±7.6
Waist circumference, cm	Baseline	75.7±7.8	81.5±9.7	85.1±13.2	90.2±8.2
	Follow-up	80.6±8.6	88.1±8.7	87.8±13.0	96.2±10.1
HDL cholesterol, mmol/L	Baseline	1.48±0.35	1.44±0.33	1.12±0.26	1.09±0.25
	Follow-up	1.81±0.42	1.63±0.39	1.54±0.32	1.36±0.36
Triglycerides, mmol/L	Baseline	0.9 [0.7; 1.3]	1.2 [0.8; 1.5]	1.7 [1.1; 2.1]	1.8 [1.5; 2.9]
	Follow-up	0.8 [0.7; 1.1]	1.5 [1.1; 2.1]	1.0 [0.7; 1.2]	1.6 [1.1; 2.4]
Glycated hemoglobin, %	Baseline	5.2±0.4	5.3±0.4	5.4±0.4	5.5±0.4
	Follow-up	5.3±0.2	5.7±0.9	5.4±0.2	5.6±0.3
Other risk factors					
Body mass index, kg/m ²	Baseline	23.2±2.6	24.9±2.8	26.3±3.7	28.0±3.0
	Follow-up	23.6±2.9	25.9±2.6	26.4±3.9	29.2±4.0
LDL-cholesterol, mmol/L	Baseline	2.89±0.80	3.24±0.84	3.33±0.93	3.42±0.88
	Follow-up	2.70±0.74	2.89±0.94	3.25±0.93	2.94±0.78
Smokers, %	Baseline	19.8	19.4	17.4	43.8
	Follow-up	12.1	22.6	17.4	28.1
Alcohol drinkers, %	Baseline	89.4	93.5	91.3	78.1
	Follow-up	92.8	93.5	95.7	81.3
Physical activity, 10 ³ METs/wk	Baseline	4.2 [2.6-6.3]	4.1 [2.2-6.4]	4.1 [3.4-6.7]	4.0 [2.6-6.5]
	Follow-up	2.7 [2.3-3.1]	2.6 [2.3-3.1]	2.6 [2.4-3.3]	2.7 [2.1-3.1]
Anti-hypertensive treatment, %	Baseline	1.0	0.0	4.3	9.7
	Follow-up	1.9	6.5	0.0	18.9
Carotid artery properties					
Inter-adventitial diameter, mm	Baseline	6.81±0.55	7.08±0.46	7.00±0.57	7.07±0.83
	Follow-up	7.01±0.62	7.31±0.56	7.20±0.55	7.51±1.00
Lumen diameter, mm	Baseline	5.57±0.54	5.79±0.46	5.78±0.58	5.79±0.79
	Follow-up	5.71±0.62	5.95±0.50	5.91±0.52	6.11±0.92
Intima-media thickness, mm	Baseline	0.62±0.10	0.64±0.10	0.61±0.06	0.64±0.12
	Follow-up	0.65±0.11	0.68±0.14	0.64±0.11	0.70±0.13
Wall cross-sectional area, mm ²	Baseline	12.1±2.3	13.0±2.2	12.2±1.6	12.9±3.2
	Follow-up	13.0±2.6	14.2±3.3	13.3±2.7	15.2±4.2
Circumferential wall tension, kPa	Baseline	29.9±4.7	32.7±4.4	33.2±5.4	34.8±7.4
	Follow-up	31.3±5.6	35.8±4.9	34.0±4.1	36.7±7.5
Circumferential wall stress, kPa	Baseline	49.1±11.4	52.1±10.8	55.5±12.5	55.9±14.2
	Follow-up	49.9±13.1	54.4±11.0	54.0±10.0	53.6±12.9
Young's elastic modulus, 10 ³ •kPa	Baseline	0.43±0.12	0.46±0.13	0.46±0.15	0.50±0.14
	Follow-up	0.44±0.15	0.52±0.14	0.50±0.14	0.56±0.20

Data are percentage, mean ± SD or median [inter-quartile range]

Table 2. Comparison of carotid artery properties across categories of changes in metabolic syndrome status

Dependent variable	Independent variable: Δ MetS status	Baseline		Follow-up		6-year Δ	
		β	95%CI	β	95%CI	β	95%CI
IAD, mm	incident	0.202	-0.001; 0.405	0.247	0.045; 0.448*	0.045	-0.097; 0.186
	recovery	0.137	-0.094; 0.368	0.139	-0.093; 0.371	0.002	-0.159; 0.163
	persistent	0.209	0.007; 0.410*	0.477	-0.278; 0.677†	0.268	0.128; 0.408†
LD, mm	incident	0.158	-0.039; 0.353	0.187	-0.009; 0.382	0.029	-0.121; 0.179
	recovery	0.164	-0.059; 0.388	0.152	-0.072; 0.377	-0.012	-0.183; 0.158
	persistent	0.183	0.002; 0.364*	0.374	0.180; 0.567†	0.191	0.042; 0.339†
IMT, mm	incident	0.020	-0.021; 0.061	0.029	-0.012; 0.070	0.009	-0.032; 0.050
	recovery	-0.017	-0.064; 0.030	-0.010	-0.057; 0.037	0.007	-0.040; 0.054
	persistent	0.010	-0.031; 0.051	0.051	0.011; 0.092*	0.041	0.001; 0.082*
CSA _{IMT} , mm ²	incident	0.78	-0.18; 1.73	1.09	0.14; 2.05*	0.33	-0.53; 1.18
	recovery	-0.04	-1.13; 1.06	0.14	-0.96; 1.23	0.17	-0.80; 1.14
	persistent	0.63	-0.33; 1.58	2.03	1.05; 3.00†	1.43	0.59; 2.28†
CWT, kPa	incident	2.22	0.52; 3.92*	3.97	2.28; 5.67†	1.75*	0.29; 3.21
	recovery	3.01	1.07; 4.96†	2.11	0.16; 4.06*	-0.90	-2.57; 0.76
	persistent	5.11	3.39; 6.84†	5.49	3.76; 7.22†	0.38	-1.11; 1.87
CWS, kPa	incident	1.77	-2.46; 5.99	3.75	-0.57; 7.84	1.98	-2.28; 6.24
	recovery	5.77	0.94; 10.58*	3.52	-1.31; 8.37	-2.25	-7.10; 2.60
	persistent	7.21	2.91; 11.51†	4.41	0.09; 8.73*	-2.80	-7.11; 1.52
YEM, 10 ³ •kPa	incident	0.015	-0.037; 0.067	0.068	0.016; 0.120*	0.050	-0.008; 0.109
	recovery	0.018	-0.041; 0.077	0.049	-0.011; 0.109	0.031	-0.038; 0.098
	persistent	0.060	0.007; 0.113*	0.117	0.066; 0.168†	0.048	-0.011; 0.107

β , regression coefficient: indicates differences in carotid artery property as compared with referent group (i.e. never); CI, confidence interval;

IAD, inter-adventitial diameter; LD, lumen diameter; IMT, intima-media thickness; CSA_{IMT}, wall cross-sectional area; CWT, circumferential wall tension; CWS, circumferential wall stress; YEM, Young's elastic modulus;

* $p < 0.05$, † $p < 0.01$, ‡ $p < 0.001$; all data are adjusted for age, sex, body height and (changes in) smoking status, alcohol consumption, physical activity, LDL-cholesterol and use of anti-hypertensive medication.

Associations Between (Changes in) MetS Traits and (Changes in) Carotid Properties

The associations of each trait of the MetS and their changes (as continuous variables) with (changes in) carotid artery properties are shown in **Table 3** and can be summarized as follows. At baseline, higher levels of IAD and LD were primarily determined by higher levels of waist circumference, whereas higher levels of CWT, CWS, and YEM were determined by both higher levels of waist and BP; none of the traits of the MetS were associated with increased IMT, however. During the course of follow-up, increases in waist circumference also determined steeper increases in IAD, LD, and YEM, and increases in BP were main determinants of increases in these properties but also of CWT and CWS. Interestingly, increases in IMT were determined only by increases in HbA1c, which was

also implicated in steeper increases in IAD and CSA_{IMT}. Changes in blood lipids did not affect changes in arterial properties.

Table 3. Associations between (changes in) each metabolic syndrome trait and (changes in) carotid artery properties

Independent variable: MetS trait	Time	Dependent variable						
		IAD (mm)	LD (mm)	IMT (mm)	CSA _{IMT} (mm ²)	CWT (kPa)	CWS (kPa)	YEM (10 ³ •kPa)
Systolic blood pressure	Baseline	-0.005	-0.016	0.008	0.15	2.62‡	3.81‡	0.039‡
	Follow-up	0.091‡	0.070‡	0.015‡	0.52‡	3.51‡	4.30‡	0.078‡
	Δ per year	0.016‡	0.014‡	0.001	0.06*	0.15‡	0.08	0.006‡
Diastolic blood pressure	Baseline	0.035	0.018	0.010	0.27*	2.74‡	3.85‡	0.040‡
	Follow-up	0.094‡	0.085‡	0.008	0.36‡	3.68‡	5.23‡	0.074‡
	Δ per year	0.010*	0.011‡	-0.000	0.02	0.16‡	0.23*	0.006‡
Waist circumference	Baseline	0.066*	0.058*	0.005	0.23	1.19‡	1.81‡	0.019*
	Follow-up	0.118‡	0.102‡	0.009	0.46‡	1.51‡	1.74‡	0.045‡
	Δ per year	0.009*	0.007*	0.001	0.04	0.05	-0.01	0.004‡
HDL-cholesterol	Baseline	-0.018	-0.021	0.001	-0.01	-0.43	-0.78	-0.008
	Follow-up	-0.002	-0.012	0.004	0.05	-0.39	-0.75	-0.011
	Δ per year	0.003	0.002	0.001	0.01	0.01	0.00	-0.000
Ln-triglycerides	Baseline	-0.005	0.005	-0.003	-0.06	0.58*	1.23*	0.014
	Follow-up	0.040	0.053*	-0.004	0.03	0.60‡	1.13	0.023‡
	Δ per year	0.008	0.008	-0.000	0.02	0.00	-0.02	0.002
Glycated hemoglobin	Baseline	-0.034	-0.014	-0.007	-0.22	-0.19	0.27	0.000
	Follow-up	0.016	0.003	0.005	0.13	0.10	-0.27	0.005
	Δ per year	0.009*	0.007	0.002*	0.06*	0.05	-0.09	0.001

Data are standardized regression coefficients and indicate the differences in carotid artery property per SD increase in MetS trait; this enables comparison of the strength of the associations with carotid properties between traits; CI, confidence interval;

IAD, inter-adventitial diameter; LD, lumen diameter; IMT, intima-media thickness; CSA_{IMT}, wall cross-sectional area; CWT, circumferential wall tension; CWS, circumferential wall stress; YEM, Young's elastic modulus;

*p<0.05, ‡p<0.01, †p<0.001; all data are adjusted for age, sex, body height and (changes in) smoking status, alcohol consumption, physical activity, LDL-cholesterol and use of anti-hypertensive medication.

Additional Analyses

When, as suggested (23), CWT and CWS were calculated at levels of DBP instead of MAP (because IMT and IAD are measured in diastole), we observed essentially the same patterns of changes and differences across groups as reported above (please see **Figure S2**).

Discussion

The main finding of our study is that individuals with persistent MetS, as compared with individuals who never had the MetS, displayed significantly steeper increases in IMT and CSA_{IMT} during the course of follow-up that were preceded by elevated levels of IAD, LD, YEM, and CWT and CWS already at baseline. This suggests that, among young individuals without overt signs of atherosclerosis, arterial enlargement and stiffening may precede and favor subsequent wall thickening to restore normal levels of wall tensile stress. Indeed, during the course of follow-up, IAD and LD increased further, and CWS tended to decrease among individuals with persistent MetS, although it remained significantly higher than that observed in individuals who never developed the MetS. Therefore, this pattern of arterial outward remodeling remained, to an important extent, maladaptive. Notably, in individuals who recovered from the MetS, all of the carotid artery properties were restored to levels that no longer differed from those who never developed the MetS. This is an important observation, because it points toward the reversibility of a pathobiological process that, if not interrupted in time, may carry adverse health consequences, such as stroke. To our knowledge, this is the first study that has examined, with a truly longitudinal design, the life course and temporal relationships between carotid artery structural and functional properties and local hemodynamic factors as a function of changes in the MetS. Being conducted among apparently healthy and young adults, our findings likely describe the early stages of accelerated arterial aging related to the MetS.

Changes in carotid IMT and distension as a function of changes in MetS over the course of 6 years among apparently healthy adults of comparable age have been reported recently in a large ($n=1673$), population-based study (24). Our findings are in agreement with this study, regarding the following: 1. the rates of changes in MetS status (never [74% versus 71%], incident [12% versus 11%], recovery [4% versus 8%], and persistent [10% versus 11%], respectively); 2. the patterns of changes in MetS traits (eg, steeper increase in DBP than SBP), even those that, at first glance, could seem counterintuitive (eg, overall increases in mean levels of high-density lipoprotein cholesterol with aging); and 3. the patterns of changes in IMT that were also steeper in those with persistent MetS only. We, thus, believe our findings, despite having been obtained with an adapted criterion to define MetS and in a smaller sample, do reflect true MetS-related arterial changes that occur in the course of young adult life. Data on concomitant changes in arterial diameter and hemodynamic forces were not provided in that study, however, thus not enabling the appreciation of MetS-related arterial remodeling processes.

Figure. Trajectories of changes in carotid artery properties by categories of change in metabolic syndrome status. **A**, IAD indicates interadventitial diameter. **B**, LUMEN D indicates lumen diameter. **C**, IMT indicates intima-media thickness. **D**, CSA_{IMT} indicates wall cross-sectional area. **E**, CWT indicates circumferential wall tension. **F**, CWS indicates circumferential wall stress. **G**, YEm indicates Young's elastic modulus. All data are adjusted for sex, height and (changes in) age, smoking status, alcohol consumption, physical activity, low-density lipoprotein cholesterol, and use of antihypertensive medication; exact magnitude and statistical significance of differences between groups are given in Table 2. o, never; •, incident; □, recovery; ■, persistent

Our study investigated these variables and raised the question of whether increases in IMT in the context of persistent MetS reflect atherosclerosis, concluding that wall thickening could primarily reflect an adaptive mechanism that attempted to restore local hemodynamic conditions to an equilibrium rather than atherosclerosis, per se. Indeed, it has been proposed that, at levels < 0.9 mm, increases in IMT may reflect adaptive arterial remodeling (6,25). Still, because the observed pattern of outward remodeling was maladaptive, our findings may have implications for cardiovascular risk, particularly stroke. Indeed, 3 different patterns of arterial remodeling have been identified: inward (decrease in LD resulting from greater increase in IMT than IAD), outward (increase in LD because of greater change in IAD than IMT), and compensatory (LD preservation despite changes in IAD and IMT) (26). Of these, inward and outward have been linked to stable and unstable plaque formation, respectively (26,27). In addition, among several carotid geometric patterns, a pattern of hypertrophy with dilation, which is, thus, equivalent to the outward remodeling described in the present study, was also linked to the highest incidence of cardiovascular disease among elderly participants of the Cardiovascular Health Study (28). Although the extent to which young adults with the MetS showing signs of maladaptive outward remodeling are more likely to develop such kind of atherosclerotic complications later in life is not known, these observations emphasize the

clinical relevance of our findings. It further stresses the potential need for early preventive measures, because we observed that recovery from MetS led to reversibility of maladaptive outward remodeling. This resulted from beneficial changes in MetS traits, in particular, DBP, waist circumference, and HbA1c (**Table S3** and **Figure S3**), all of which contributed also to arterial adaptations (**Table 3**).

Because of the longitudinal design, we obtained a better insight into the sequence of carotid structural and functional adaptations related to the MetS. The observation that persistent MetS was not only associated with increased IAD and LD but also YEM (a marker of the elastic properties of the arterial wall material) already at baseline, suggests that loss of elasticity at the level of the media layer (ie, arteriosclerosis) could explain the arterial enlargement of individuals with the MetS. Consequently, the subsequent increases in IMT and CSA_{IMT} could reflect wall changes occurring primarily at the level of the media rather than the intima (atherosis) layer.

Increases in CSA_{IMT} (although not IMT) were related to changes in SBP, and increases in IAD and LD were associated with both changes in SBP and DBP but more strongly with the former than the latter (**Table 3**). This points toward a stronger impact of the pulsatile component of BP on arterial remodeling. Indeed, when we additionally examined the extent to which the steeper increases in IAD, LD, IMT, and CSA_{IMT} observed among individuals with persistent MetS could be explained by concomitant changes in BP, we observed that these were not affected when further adjusted for changes in DBP but were somewhat attenuated when adjusted for changes in local pulse pressure (**Table S4**). Transmural pressure exerts a fatiguing effect on the load bearing elements of the arterial wall (ie, elastin and collagen), leading to degenerative changes and fracture of the extracellular matrix component that result in arterial enlargement (23). Nevertheless, the steeper increases in IAD, LD, IMT, and CSA_{IMT} in individuals with persistent versus never MetS remained statistically significant after adjustments for BP, suggesting that other factors may be involved. Indeed, we observed that increases in these arterial properties were also determined by changes in HbA1c. This could reflect underlying cross-links between molecules in the arterial wall because of advanced-glycation end products, thereby contributing directly to accelerated arterial stiffening, as commonly observed among patients with (pre)diabetes mellitus (3). However, HbA1c was not related to YEM, a marker of the intrinsic elastic wall material. In addition, metabolic factors related to central adiposity, yet another MetS trait related to changes in IAD, LD, and YEM, can also play an additional disrupting role in the intricate process of arterial remodeling. These may include adipocytokine or systemic inflammatory factors, oxidative stress, vascular smooth muscle cell growth, and stimulation of the sympathetic nervous system (3,16). All together these observations suggest that clustering of elevated BP, abdominal obesity, and

impaired glucose metabolism may constitute a phenotype with the most adverse impact on arterial properties. This is in line with earlier cross-sectional observations (9), but because of the relatively small sample of the present study, we could not examine arterial changes associated with specific clusters of MetS traits further.

There are additional limitations to our study. First, ultrasound does not distinguish the intima from the media layers of the carotid wall, thus not enabling full insight on whether wall thickening related to MetS was particularly associated with atherosclerosis (intima) or arteriosclerosis (media). Second, IMT was measured at one arterial site, and although we took measures to ensure that follow-up examinations did not differ from baseline in this regard, at both time points, our measures do not capture possible variability in IMT along the arterial wall. Third, although good levels of reproducibility were obtained between observers involved in the baseline (I.F.) and the follow-up (F.S.) examinations, data on changes in arterial properties may have been obtained with some error. If anything, this may have led to an underestimation of the associations reported herein, because both at baseline and at follow-up observers were unaware of participants' metabolic risk profiles (ie, nondifferential error). Fourth, the prevalence of the MetS as reported herein may not compare directly with that obtained with standard definitions, because it was estimated on the basis of risk factor distribution within the study population. Still, it served the purpose of identification of a phenotype (clustering of elevated MetS traits) and its change over time, which is suitable for the study of an etiologic research question, that is, its association with arterial remodeling. Last, our findings were obtained in relatively young and apparently healthy white adults and should be interpreted with caution with regard to older and higher-risk populations and other ethnicities.

Perspectives

We identified a pattern of outward carotid remodeling (ie, diameter widening and wall thickening, in this order) among individuals with persistent versus never MetS over the course of 6-year follow-up. This process was maladaptive because, despite remodeling, wall tensile stress was not restored to levels comparable to those who never had the MetS. Importantly, reversibility of maladaptive outward remodeling was achieved by recovery from the MetS. Maladaptive carotid remodeling may carry important implications for cardiovascular risk, particularly stroke. Prospective studies investigating the link between patterns of arterial remodeling in young adults with future cardiovascular outcome are further needed, to decide on the usefulness of screening and

implementation of appropriate preventive measures likely to deter related cardiovascular sequelae.

Novelty and Significance

What Is New?

- We studied changes in carotid structural, functional, and local hemodynamic factors as a function of changes in MetS status over the course of 6-years follow-up.
- Given the longitudinal design, we could examine the temporal relationships between diameter and wall thickening and concomitant hemodynamic factors.

What Is Relevant?

- We observed the development of a pattern of maladaptive outward carotid remodeling among individuals with persistent MetS and a similar trend among individuals with incident MetS.
- Recovery from the MetS restored initial adverse carotid adaptations to levels comparable to those who never developed the MetS. Wall thickening was preceded by wall widening.

Summary

- The development of maladaptive outward remodeling may explain the increased risk of cardiovascular disease, in particular, stroke, in individuals with the MetS.

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-ONLINE SUPPLEMENT

CLUSTERING OF METABOLIC SYNDROME TRAITS IS ASSOCIATED WITH MALADAPTIVE CAROTID REMODELING AND STIFFENING: A 6-YEAR LONGITUDINAL STUDY

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METHODS

Carotid artery properties were assessed according to guidelines for user procedures and with the use of reproducible and valid methods and devices [Ultrasound scanner + Wall Track System 2 (WTS2), Pie Medical, Maastricht, the Netherlands] (1,2). Measures obtained in the year 2006 (by FS) (3) followed the exact same protocol as conducted in 2000 (by IF, described in detail before(4-7)), with good levels of interobserver reproducibility (Table S1) (3).

Briefly, all subjects abstained from smoking and caffeine-containing beverages on the day the

measurements were performed, and these started after subjects had been resting in supine position for 15 min in a quiet, temperature-controlled, room. We obtained measurements of inter-adventitial

diameter (IAD, in diastole), distension (ΔD , i.e. the systolic-diastolic variation in IAD) and intima-media thickness (IMT) as detailed elsewhere (1). The right common carotid artery was measured ~10 mm proximal to the beginning of the bulb; placement of the M-line was video-recorded and a print-out was obtained in order to ensure that follow-up measures, as obtained in 2006, were obtained at the exact same site (3). The means of 3 measurements of IAD, ΔD and IMT were used in the analyses.

Statistical analyses

A simplification (without covariates adjustments) of the longitudinal models used in the present study is presented below (8):

Dependent variable = $\beta_0 + (\beta_1 \times \text{incident MetS}) + (\beta_2 \times \text{recovery MetS}) + (\beta_3 \times \text{persistent MetS}) + (\beta_4 \times \text{time}) + (\beta_5 \times \text{incident MetS} \times \text{time}) + (\beta_6 \times \text{recovery MetS} \times \text{time}) + (\beta_7 \times \text{persistent MetS} \times \text{time})$

...where:

β_0 = intercept (level of dependent variable in the never MetS group at time=0);

β_1 = difference in dependent variable between incident MetS and never MetS groups at time=0;

β_2 = difference in dependent variable between recovery MetS and never MetS groups at time=0;

β_3 = difference in dependent variable between persistent MetS and never MetS groups at time=0;

β_4 = change in dependent variable per year in the never MetS group;

β_5 = difference in 6-yr change in the dependent variable between incident MetS and never MetS groups;

β_6 = difference in 6-yr change in the dependent variable between incident MetS and never MetS groups;

β_7 = difference in 6-yr change in the dependent variable between incident MetS and never MetS groups;

The value of the dependent variable at follow-up (i.e. t=6) for each group, can be estimated as:

- $\beta_0 + (6 \times \beta_4)$ in the never MetS group;

- $\beta_1 + (6 \times \beta_4) + \beta_5$ in the incident MetS;

- $\beta_2 + (6 \times \beta_4) + \beta_6$ in the recovery MetS group; and by

- $\beta_3 + (6 \times \beta_4) + \beta_7$ in the persistent MetS group;

Categories of change in MetS status was treated as dummy variable with never MetS as referent group.

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Table S1. Inter-observer reproducibility of carotid artery properties

Carotid artery property	Observers				Differences between observers					
	IF		FS		FS-IF					
	Mean	SD	Mean	SD	Mean	SE	95%CI	p-value	ICC	CV%
Diameter, μm	6,475	481	6,493	451	16	88	-156; 188	0.859	0.93	2.7
Distension, μm	550	206	523	204	-28	26	-78; 22	0.278	0.97	9.5
IMT, μm	622	68	585	69	-35	20	-74; 4	0.080	0.77	6.2

Data refers to a pilot-reproducibility study conducted prior to the follow-up examination between the observers who collected the baseline (IF) and the follow-up (FS) arterial data [n=8 (4 women); each observer obtained 3 repeated measures of each property on each subject]); ICC, intra-class correlation coefficient; CV, coefficient of variation.

Table S2. Sex-specific cut-off values for each trait of the metabolic syndrome corresponding to the lower bound of the highest quartile (lowest for HDL-cholesterol)

Trait of the metabolic syndrome	Men	Women
Systolic/diastolic blood pressure, mm Hg	$\geq 128/72$	$\geq 117/67$
Waist circumference, cm	≥ 90.1	≥ 76.5
HDL cholesterol, mmol/L	≤ 1.02	≤ 1.35
Triglycerides, mmol/L	≥ 1.9	≥ 1.3
Glycated hemoglobin, %	≥ 5.6	≥ 5.5

FIGURE S1 Distribution of risk factor sums in the AGAHLs at baseline and at follow-up; bars represent observed frequencies and line represents the expected frequencies (from the binomial probability equations).

Table S3. Comparison of levels of metabolic syndrome's traits across categories of changes in metabolic syndrome status

Dependent variable	Independent variable: Δ MetS status	Baseline		Follow-up		6-year Δ	
		β	95%CI	β	95%CI	β	95%CI
SBP, mm Hg	incident	5.4	2.1; 8.8 [†]	10.1	5.2; 14.9 [‡]	4.7	0.2; 9.1*
	regressed	6.5	3.8; 9.3 [‡]	3.2	-0.9; 7.2	-3.4	-8.2; 1.5
	persistent	13.4	8.3; 18.5 [‡]	11.2	5.2; 17.2 [‡]	-2.2	-6.9; 2.5
DBP, mm Hg	incident	3.9	1.7; 6.2 [†]	6.6	4.1; 9.1 [‡]	2.7	0.3; 5.0*
	regressed	5.3	2.9; 7.6 [‡]	2.5	0.1; 4.9*	-2.8	-5.7; 0.1
	persistent	8.2	5.4; 11.0 [‡]	6.1	3.1; 9.2 [‡]	-2.1	-4.5; 0.4
WAIST, cm	incident	3.9	1.7; 6.1 [†]	6.2	4.2; 8.1 [‡]	2.3	0.7; 3.9 [†]
	regressed	8.4	4.3; 12.4 [‡]	6.0	1.9; 10.1 [†]	-2.4	-4.0; -0.8 [†]
	persistent	13.9	11.3; 16.5 [‡]	15.9	13.1; 18.7 [‡]	2.0	0.1; 4.0*
HDL, mmol/L	incident	0.01	-0.10; 0.12	-0.14	-0.27; -0.01*	-0.15	-0.26; -0.04 [†]
	regressed	-0.32	-0.43; -0.22 [‡]	-0.23	-0.36; -0.10 [‡]	0.09	-0.01; 0.19
	persistent	-0.35	-0.43; -0.26 [‡]	-0.42	-0.53; -0.30 [‡]	-0.07	-0.16; 0.01
TRIG, mmol/L	incident	1.09	0.93; 1.28	1.65	1.37; 1.99 [‡]	1.51	1.29; 1.78 [‡]
	regressed	1.67	1.43; 1.94 [‡]	1.03	0.89; 1.20	0.62	0.50; 0.77 [‡]
	persistent	2.00	1.69; 2.38 [‡]	1.82	1.50; 2.22 [‡]	0.92	0.78; 1.09
HbA1c, %	incident	0.14	-0.01; 0.29	0.36	0.03; 0.68*	0.22	-0.12; 0.55
	regressed	0.27	0.11; 0.42 [†]	0.03	-0.05; 0.11	-0.23	-0.37; -0.09 [†]
	persistent	0.29	0.13; 0.45 [†]	0.31	0.20; 0.41 [‡]	0.02	-0.12; 0.15

β , regression coefficient: indicates differences in levels of each trait of the metabolic syndrome as compared with referent group (i.e. never); CI, confidence interval;

SBP, systolic blood pressure; DBP, diastolic blood pressure; WAIST, waist circumference, HDL, high-density lipoprotein cholesterol; TRIG, triglycerides; HbA1c, glycated hemoglobin;

* $p < 0.05$, $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$; all data are adjusted for age, sex, body height and (changes in) smoking status, alcohol consumption, physical activity, low-density lipoprotein cholesterol and use of anti-hypertensive medication.

Note that because triglycerides were $\log_e$ transformed, differences (vs. reference group) are expressed as ratios.

Table S4. 6-year changes in carotid structure properties by groups of change in MetS status, after further adjustments for changes in blood pressure

Dependent variable	Independent variable: Δ MetS status	As reported in our manuscript§		+ adjustment for changes in DBP		+ adjustment for changes in MBP		+ adjustment for changes in local PP	
		β	95%CI	β	95%CI	β	95%CI	β	95%CI
IAD, mm	incident	0.045	-0.097; 0.186	0.006	-0.142; 0.151	-0.001	-0.147; 0.145	-0.031	-0.172; 0.109
	recovery	0.002	-0.159; 0.163	0.006	-0.158; 0.171	-0.001	-0.163; 0.162	-0.005	-0.163; 0.153
	persistent	0.268	0.128; 0.408†	0.254	0.106; 0.403†	0.245	0.097; 0.394†	0.218	0.078; 0.359†
LD, mm	incident	0.029	-0.121; 0.179	0.018	-0.121; 0.179	-0.021	-0.175; 0.134	0.027	-0.123; 0.178
	recovery	-0.012	-0.183; 0.158	0.006	-0.183; 0.158	-0.028	-0.200; 0.145	-0.007	-0.177; 0.162
	persistent	0.191	0.042; 0.339†	0.203	0.054; 0.352†	0.155	0.002; 0.308*	0.158	0.008; 0.309*
IMT, mm	incident	0.009	-0.032; 0.050	0.013	-0.029; 0.055	0.009	-0.033; 0.051	0.001	-0.040; 0.042
	recovery	0.007	-0.040; 0.054	0.017	-0.031; 0.064	0.013	-0.035; 0.060	0.005	-0.042; 0.051
	persistent	0.041	0.001; 0.082*	0.053	0.010; 0.095*	0.048	0.006; 0.091*	0.034	-0.005; 0.020
CSA _{IMT} , mm ²	incident	0.33	-0.53; 1.18	0.30	-0.58; 1.18	0.20	-0.68; 1.08	0.13	-0.71; 0.98
	recovery	0.17	-0.80; 1.14	0.35	-0.64; 1.34	0.25	-0.73; 1.23	0.14	-0.82; 1.10
	persistent	1.43	0.59; 2.28†	1.60	0.71; 2.49†	1.48	0.59; 2.37†	1.16	0.32; 2.01†

β , regression coefficient; indicates differences in carotid artery property as compared with referent group (i.e. never); CI, confidence interval;

IAD, inter-adventitial diameter; LD, lumen diameter; IMT, intima-media thickness; CSA_{IMT}, wall cross-sectional area; CWT, circumferential wall tension; CWS, circumferential wall stress; YEM, Young's elastic modulus;

DBP, diastolic blood pressure; MBP, mean blood pressure; PP, pulse pressure;

*p<0.05, †p<0.01, ‡p<0.001; § as reported in Table 2 of our manuscript; all data are adjusted for sex, body height and (changes in) age, smoking status, alcohol consumption, physical activity, LDL-cholesterol and use of anti-hypertensive medication.

FIGURE S2 Trajectories of changes in A) circumferential wall tension (CWT) and B) circumferential wall stress (CWS) when both hemodynamic variables were estimated at diastolic levels of blood pressure; Data are adjusted for sex, body height and (changes in) age, smoking habits, alcohol consumption, physical activity, LDL-cholesterol and use of anti-hypertensive medication

FIGURE S3. Trajectories of changes in metabolic syndrome traits by categories of change in metabolic syndrome status; A: systolic blood pressure (SBP); B: diastolic blood pressure (DBP); C: waist circumference (WAIST); D: HDL-cholesterol (HDL); E: triglycerides (TRIG); and F: glycated hemoglobin (HbA1c). Data are adjusted sex, body height and (changes in) age, smoking status, alcohol consumption, physical activity, LDL-cholesterol and use of anti-hypertensive medication (the exact magnitude and statistical significance of differences between groups are given in Table S3).

Chapter 6

Body composition as determinant of thrombin generation in plasma.

The Hoorn Study.

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Abstract

Objective —The association between obesity and cardiovascular disease and venous thromboembolism might, at least partially, be explained by a hypercoagulable state. The extent to which body fat mass and its distribution contribute to a hypercoagulable state is unknown. In this study, we investigated the association between body composition and thrombin generation and evaluated the potential mediating role of low-grade inflammation.

Methods and Results—We studied 586 individuals from the Hoorn Study (mean age, 69.7 ± 6.5 years, 298 women) in whom body composition was assessed by whole body dual-energy absorptiometry. Thrombin generation was measured using the Calibrated Automated Thrombogram. Multiple regression analyses showed a positive association between total body fat and thrombin generation in women but not in men. In addition, detailed analyses of regional body composition showed that central but not peripheral fat mass was associated with greater thrombin generation and that there was a trend toward an inverse association with peripheral lean mass. The reported positive associations were partially attenuated by low-grade inflammation, however.

Conclusion — Body fat mass, in particular a central pattern of fat distribution, is associated with higher levels of thrombin generation in elderly women but not in men. This association may partially be explained by adiposity-related low-grade inflammation, but this hypothesis needs to be further investigated in mechanistic/prospective studies.

Introduction

Obesity, in particular central obesity, is associated with cardiovascular disease (CVD) (1,2). Recent evidence also suggests an association between (central) obesity and incident venous thromboembolism (3,4). One proposed mechanism that could explain these associations, at least in part, is a hypercoagulable state in obese individuals (5). Indeed, thrombin is one of the key players in the coagulation cascade (6), and in vivo markers of thrombin generation have been positively associated with waist circumference in postmenopausal women (7).

Recently, several new methods have become available that quantitatively measure thrombin generation in vitro after activation of the coagulation cascade with tissue factor (TF), phospholipids, and calcium chloride (CaCl_2) (8). The Calibrated Automated Thrombogram (CAT) method is one such method (9). Specifically, it generates a thrombin generation curve that mimics the overall plasma coagulability potential when a thrombogenic stimulus appears (**Figure 1A**). This curve is characterized by a lag (initiation) phase, followed by a thrombin burst (propagation), which is then eventually completely inhibited by plasma protease inhibitors (termination). The endogenous thrombin potential (ETP), or the area under this curve, represents the total amount of active thrombin that is formed after activation of the coagulation cascade. The ETP has been shown to be a good indicator of a hypercoagulable state in, for example, individuals using oral contraceptives or with (prior) venous thromboembolism or circulating lupus anticoagulants (9–12).

Evidence so far linking obesity to an individual's coagulation potential assessed by this new method is very scarce and confined to children and adults with morbid obesity (13,14). In addition, the extent to which total fat mass and its distribution contribute to or protect against a hypercoagulable state is not known. It is also unknown what adiposity-related underlying mechanisms may explain any such associations, but low-grade inflammation is a likely candidate in this regard. Indeed, levels of high-sensitivity C-reactive protein (hsCRP) are increased in overweight and obese individuals (15), and in vivo, C-reactive protein infusion has been associated with increased thrombin generation (assessed by activation markers) (16).

In view of these considerations, we investigated, in a large population-based cohort, the association between body composition and thrombin generation in plasma and evaluated whether any such associations were independent of other “traditional” cardiovascular risk factors, such as hypertension, dyslipidemia, smoking, and glucose metabolism status. In addition, we evaluated the potential “mediating” role of hsCRP in any such associations.

Methods

Study Population

The Hoorn Study is a population-based cohort study of glucose metabolism and its complications, which started in 1989, as described in detail elsewhere (17–19). Briefly, a random sample of 50- to 75-year-old subjects was taken from the population register of Hoorn (59,000 inhabitants) in the Netherlands. Of the 3553 subjects invited, 2540 (71.5%) agreed to participate, of whom 56 were excluded from analyses because they were nonwhite. The original Hoorn Study cohort thus consisted of 2484 subjects (18). Between 1996 and 1998, a 2nd examination (1st follow-up) was carried out. From the initial cohort, 150 people had died and 108 had moved out of Hoorn before 1996. One hundred forty other people were not invited because of logistical reasons. Of the remaining 2086 people who were invited for the follow-up examination, 1513 (72.5%) participated (17). In 2000–2001, a 3rd examination was carried out among surviving participants who gave their permission to be recontacted. Specifically, we invited all participants who had diabetes, as determined by a 75-g oral glucose tolerance test or by diabetes treatment ($n=176$), and a random sample of participants who had normal ($n=705$) or impaired glucose tolerance ($n=193$) at the 2nd examination. Of the 1074 individuals invited, 648 (60.3%) participated. The main reasons for nonparticipation in the 2000–2001 follow-up examination were lack of interest (30%) or comorbidity (23%). Other reasons were high age (7%), unwillingness to travel (6%), participation considered too time-consuming (6%), and miscellaneous reasons (15%); 13% gave no reason (19).

Detailed measurements of participants' body composition by means of dual-energy x-ray absorptiometry (DXA) and thrombin generation parameters were obtained, for the first time, during the 3rd examination. Therefore, for the present study, cross-sectional analyses of data from this examination were conducted. We excluded 62 participants from the analyses for the following reasons: use of cumarin derivatives ($n=24$), and/or use of hormonal replacement therapy ($n=2$), and/or missing data on thrombin generation tests ($n=31$), and/or missing data on DXA measurements ($n=25$). The present study therefore consisted of 586 individuals.

The Ethical Review Committee of the Vrije Universiteit University Medical Centre approved the study protocol, and all participants gave their written informed consent.

Anthropometrics/Body Composition

Weight and height were measured while participants were barefoot, wearing only light clothing. Body mass index (BMI) was calculated as weight (kg) divided by height (m) squared. Waist circumference was measured at the level midway between the lowest rib

margin and the iliac crest, and hip circumference was measured at the widest level over the greater trochanters.

A whole body DXA scan was performed using the fan beam technology (QDR-2000, software version 7.20D, Hologic, Brussels, Belgium). This software provides estimates of lean tissue mass, fat mass, and bone mineral mass for the total body and for standard body regions. Regions of the head, trunk, arms, and legs were distinguished with the use of specific anatomic landmarks (19). In the analyses, we used fat and lean soft tissue mass from the trunk, arms, and legs. Peripheral fat was calculated by adding the fat mass of the legs to that of the arms, and peripheral lean mass was calculated by adding the lean mass of the legs to that of the arms (20).

Thrombin Generation Parameters

Venous blood samples were collected in 2000–2001, and 3.2% citrated platelet-poor plasmas were prepared by 2-step centrifugation: at 4000g for 15 minutes followed by centrifugation at 10 000g for 5 minutes. Plasmas were stored at -80°C until analysis in 2008 and were not thawed before then. Thrombin generation in platelet-poor plasma was measured using the CAT method (9), which uses low-affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg- AMC) to continuously monitor thrombin activity in clotting plasma. Thrombin generation was assessed according to manufacturer's instructions (Thrombinoscope BV) in a 96-well plate fluorometer (Ascent Reader, Thermolabsystems OY, Helsinki, Finland) as previously described (21). Briefly, thrombin generation was determined in the presence of: 1 pM TF and 4 μ M phospholipids in the absence and presence of 0.55 nM recombinant soluble thrombomodulin (Paion GmbH, Aachen, Germany). The 1 pM TF trigger was a commercial product (PPP Reagent Low, Thrombinoscope BV, Maastricht, the Netherlands). Three parameters were derived from these analyses: lag time (min), peak height (nM), and ETP [nM]. Finally, a thrombomodulin ratio was calculated by dividing the ETP and peak height obtained in the presence by those in the absence of thrombomodulin. Thrombomodulin activates the anticoagulant protein C pathway, and therefore this ratio represents the inhibition of thrombin generation by activation of the protein C pathway. At our laboratory, the intraassay coefficients of variation (CVs) are <5% (21), and the interassay CVs, obtained in 2 lots of normal-pool plasma in 25 independent runs during the present study, were < 11% for all thrombin generation parameters (calculated using EP Evaluator 8.0.0.90 software).

Other Measurements

Health status, medical history, medication use, and smoking habits were assessed by questionnaires (18). Systolic blood pressure (SBP) and diastolic blood pressure, levels of plasma glucose (fasting and postload), insulin, total, high- and low-density lipoprotein cholesterol, triglycerides, and hsCRP were measured as described elsewhere (19). In addition, urinary albumin-to-creatinine ratio was measured in an early morning first-voided urine sample, and accordingly, participants were classified as having microalbuminuria if the albumin-to-creatinine ratio was 2.0 to 30 mg/mmol. Hypertension was defined as SBP $\geq$ 140 mm Hg or diastolic blood pressure $\geq$ 90 mm Hg, or the use of antihypertensive drugs (22).

Prior CVD was defined when individuals had any of the following: a history of myocardial infarction, transient ischemic attack or ischemic stroke, abnormalities on a resting ECG (Minnesota codes 1.1 to 1.3, 4.1 to 4.3, 5.1 to 5.3, or 7.1), undergone coronary bypass surgery or angioplasty, peripheral arterial bypass or nontraumatic amputation, or ankle-brachial index of < 0.9 in either leg.

Statistical Analyses

Comparisons of the clinical characteristics according to sex were investigated with the use of the Student *t* test for continuous variables and χ^2 test for frequency data.

We used multiple linear regression analyses to investigate the associations between body composition and thrombin generation parameters. First, associations with DXA and anthropometric estimates of total body fat were examined. These analyses were adjusted for age, prior CVD, glucose metabolism, and smoking status (model 1) and other cardiovascular risk factors, ie, SBP (model 2), dyslipidemia (model 3), and microalbuminuria (model 4). Second, the contributions of the regional fat and lean mass, estimated by either DXA or anthropometrics, were analyzed in mutually adjusted models. In both sets of analyses, the potential mediating role of low-grade inflammation (hsCRP) was investigated by calculating the magnitude of the changes in the regression coefficients after further adjustment for this variable. All results are shown as standardized regression coefficients (β) and the respective 95% confidence intervals (CIs) and were performed in men and women separately. Assumption of linearity intrinsic to the method of data analyses adopted was checked by inspection of the residuals by predicted values scatter plots (23). We considered the stability of the models to be disturbed by multicollinearity if the tolerance was < 0.1 (23).

A 2-sided probability value of < 0.05 was considered statistically significant. All analyses were performed by using the Statistical Package for Social Sciences (SPSS) for Windows, version 15.0 (SPSS Inc., Chicago, Ill).

Results

Participants who were excluded from the analyses (n=62) were older; had a higher BMI and waist circumference; and more often had hypertension, dyslipidemia, diabetes, and prior CVD than those included (data not shown). The clinical characteristics of the participants included (n=586) are shown in **Table 1**, stratified by sex. Women had smaller waist but larger hip circumferences and higher levels of total, trunk, and peripheral fat but less lean mass than men. Men and women did not differ with respect to thrombin generation parameters.

Total Body Fat and Thrombin Generation

Total body fat percentage (as estimated by DXA) or BMI, both estimates of total body adiposity, were positively associated with lag time, peak height, and ETP in women, whereas no such associations were seen in men. These associations were independent of age, prior CVD, glucose metabolism, and smoking status (**Table 2**, model 1) and are also illustrated in **Figure 1B**, showing the differences in these parameters across categories of BMI. Additional adjustment for SBP (model 2), dyslipidemia (model 3), or microalbuminuria (model 4) only slightly attenuated the associations with lag time and peak height and ETP. The strengths of the associations were comparable for total body fat percentage (estimated by DXA) and BMI.

Regional Body Composition and Thrombin Generation

DXA

Trunk fat and peripheral fat mass were both positively associated with lag time, peak height, and ETP, again in women but not in men. Peripheral lean mass was positively associated with lag time only (**Table 3**, model 0). When adjusted for each other, trunk fat, not peripheral fat or lean masses, was the strongest correlate of lag time, peak height, and ETP (**Table 3**, model 2; **Figure 2**). Additional adjustment for SBP (model 3), dyslipidemia (model 4), or microalbuminuria (model 5) attenuated these associations only slightly. In these mutually adjusted analyses, if anything, there was a trend toward an inverse association between peripheral lean mass and peak height and ETP in women (**Table 3**, models 2 to 5; **Figure 2**), whereas no association was observed between peripheral fat and study outcomes (**Table 3**, models 2 to 5; **Figure 2**).

Table 1: Clinical characteristics of the study population

Characteristic	Men (n=288)	Women (n=298)	p-value
Age, years	69.6 ± 6.3	69.9 ± 6.6	0.542
Anthropometry			
Height, cm	176 ± 6	163 ± 6	<0.001
Weight, kg	83 ± 12	73 ± 12	<0.001
Body mass index, kg/m ²	26.9 ± 3.5	27.5 ± 4.2	0.057
Waist circumference, cm	99 ± 10	91 ± 12	<0.001
Hip circumference, cm	101 ± 7	104 ± 9	<0.001
DXA			
Total body fat, %	28 ± 7	42 ± 7	<0.001
Trunk fat mass, kg	12.7 ± 5.5	14.7 ± 5.5	<0.001
Trunk lean mass, kg	28.5 ± 3.1	20.8 ± 2.4	<0.001
Peripheral fat mass, kg	9.5 ± 2.9	14.9 ± 4.6	<0.001
Peripheral lean mass, kg	24.9 ± 3.4	17.0 ± 2.4	<0.001
Metabolic and other variables			
Fasting glucose, mmol/l	6.25 ± 1.41	6.13 ± 1.41	0.322
Glucose metabolism status, %			
NGM/IGM/DM2	46 / 31 / 23	49 / 28 / 23	0.602*
Total cholesterol, mmol/l	5.38 ± 0.98	6.09 ± 0.96	<0.001
LDL-cholesterol, mmol/l	3.46 ± 0.88	3.84 ± 0.89	<0.001
HDL-cholesterol, mmol/l	1.25 ± 0.32	1.61 ± 0.41	<0.001
Triglycerides, mmol/l	1.30 [1.00-1.70]	1.30 [0.90-1.70]	0.314
Total cholesterol/HDL ratio	4.52 ± 1.24	4.03 ± 1.22	<0.001
Lipid-lowering drugs, %	16	14	0.459
Systolic blood pressure, mmHg	142 ± 19	143 ± 22	0.838
Diastolic blood pressure, mmHg	84 ± 10	82 ± 12	0.162
Antihypertensives, %	33	36	0.491
Hypertension, %	67	64	0.775
(Micro)albuminuria, %	17	11	0.067
Platelet aggregation inhibitors, %	22	11	<0.001
Current smoker, %	17	11	0.030
Prior cardiovascular disease, %	49	48	0.812
High-sensitivity C-reactive protein, mg/l	1.95 [1.16- 4.29]	1.98 [0.90- 4.25]	0.288
Thrombin generation parameters			
<i>Analyses without thrombomodulin</i>			
Lag time, min	4.08 ± 0.92	4.00 ± 0.63	0.210
ETP, nM*min	1188 ± 234	1183 ± 203	0.808
Peak height, nM	185 ± 55	187 ± 55	0.765
<i>Analyses with thrombomodulin</i>			
Lag time, min	3.99 ± 0.74	3.96 ± 0.64	0.687
ETP, nM*min	801 ± 253	779 ± 236	0.290
Peak height, nM	162 ± 58	162 ± 58	0.870
<i>TM-ratio</i>			
ETP ^{TM+} /ETP ^{TM-}	0.66 ± 0.14	0.65 ± 0.13	0.196
Peak height ^{TM+} / Peak height ^{TM-}	0.85 ± 0.12	0.85 ± 0.09	0.963

Results are expressed as mean ± SD, frequencies (%) or medians [inter-quartile range] *p for trend

DXA: dual energy-absorptiometry; NGM: normal glucose metabolism; IGM: impaired glucose metabolism; DM2: type 2 diabetes; LDL: low-density lipoprotein; HDL: high-density lipoprotein; ETP: endogenous thrombin potential; TM: thrombomodulin

Figure 1. A, Example of a thrombin generation curve obtained by means of the CAT method in normal individuals. The parameters derived from the thrombin generation curve are: lag time (the time in which ~10nM of thrombin is formed), peak height (the maximum amount of formed thrombin), and ETP (the area under the curve). **B**, thrombin generation curves in women across categories of BMI: normal weight (BMI: 18.5-24.9 kg/m²), overweight (BMI: 25-29.9 kg/m²) and obese (BMI ≥ 30 kg/m²). Those who were overweight had 0.2%, 10.4%, and 6.3% longer/higher lag time, peak height and ETP respectively, and those with obesity had 5.2%, 14.6% and 8.4% longer/higher lag time, peak height and ETP respectively, compared with those with normal weight (*P* for trend < 0.04 for all).

Table 2. Associations of total body fat (according to DXA or anthropometrics) with thrombin generation parameters

Men							
Determinant	Model	Lag time		Peak height		ETP	
		β	95%CI	β	95%CI	β	95%CI
Total body fat % (DXA)	0	0.01	-0.11; 0.12	0.03	-0.09; 0.14	0.00	-0.12; 0.12
	1	0.01	-0.11; 0.14	0.01	-0.12; 0.13	-0.02	-0.14; 0.11
	2	0.00	-0.12; 0.13	0.01	-0.12; 0.14	-0.02	-0.14; 0.11
	3	-0.04	-0.17; 0.09	0.00	-0.13; 0.14	-0.03	-0.16; 0.11
	4	0.01	-0.12; 0.13	0.01	-0.12; 0.13	-0.02	-0.14; 0.11
Body mass index	0	-0.08	-0.19; 0.04	0.01	-0.11; 0.13	-0.03	-0.15; 0.09
	1	-0.08	-0.20; 0.05	0.01	-0.11; 0.13	-0.04	-0.16; 0.09
	2	-0.09	-0.21; 0.04	0.01	-0.11; 0.14	-0.04	-0.16; 0.09
	3	-0.13*	-0.26; -0.00	0.01	-0.12; 0.14	-0.05	-0.18; 0.08
	4	-0.07	-0.20; 0.05	0.01	-0.11; 0.13	-0.04	-0.16; 0.09
Women							
Determinant	Model	Lag time		Peak height		ETP	
		β	95%CI	β	95%CI	β	95%CI
Total body fat % (DXA)	0	0.16 [†]	0.04; 0.27	0.14*	0.03; 0.26	0.22 [‡]	0.10; 0.33
	1	0.13*	0.01; 0.25	0.13*	0.01; 0.25	0.21 [†]	0.10; 0.33
	2	0.14*	0.02; 0.26	0.11	-0.02; 0.23	0.19 [†]	0.07; 0.31
	3	0.11	-0.01; 0.22	0.13*	0.01; 0.25	0.21 [†]	0.09; 0.33
	4	0.13*	0.01; 0.25	0.13*	0.01; 0.24	0.21 [†]	0.09; 0.33
Body mass index	0	0.21 [†]	0.10; 0.32	0.14*	0.03; 0.25	0.18 [†]	0.06; 0.28
	1	0.18 [†]	0.06; 0.30	0.12	-0.01; 0.24	0.16 [†]	0.04; 0.28
	2	0.19 [†]	0.07; 0.31	0.09	-0.03; 0.21	0.14*	0.02; 0.26
	3	0.14*	0.03; 0.26	0.13*	0.01; 0.25	0.16 [†]	0.04; 0.28
	4	0.18 [†]	0.06; 0.30	0.12	-0.01; 0.24	0.16 [†]	0.04; 0.28

β , standardized regression coefficient; indicates the change in thrombin generation parameter (in SD) per 1 SD increase in determinant; CI, confidence interval; * $p < 0.05$, [†] $p < 0.01$, [‡] $p < 0.001$;

Model 0: crude;

Model 1: adjusted for age, prior CVD, glucose metabolism and smoking status;

Model 2: model 1 + SBP;

Model 3: model 1 + total cholesterol/HDL ratio, ln-triglycerides;

Model 4: model 1 + microalbuminuria.

Table 3. Associations of regional body composition (according to DXA or anthropometrics) with thrombin generation parameters

		Men						Women					
Determinant	Model	Lag time		Peak height		ETP		Lag time		Peak height		ETP	
		β	95%CI	β	95%CI	β	95%CI	β	95%CI	β	95%CI	β	95%CI
DXA													
Trunk fat mass	0	-0.01	-0.13; 0.11	0.01	-0.11; 0.12	-0.01	-0.13; 0.11	0.22 [†]	0.11; 0.33	0.13*	0.02; 0.25	0.18 [†]	0.07; 0.30
	1	-0.00	-0.13; 0.12	-0.00	-0.13; 0.12	-0.02	-0.15; 0.10	0.20 [†]	0.07; 0.32	0.11	-0.02; 0.23	0.17 [†]	0.04; 0.29
	2	0.14	-0.08; 0.36	-0.11	-0.32; 0.11	0.01	-0.21; 0.23	0.20*	0.03; 0.37	0.15	-0.03; 0.32	0.17	-0.01; 0.34
	3	0.12	-0.11; 0.34	-0.11	-0.33; 0.12	0.01	-0.22; 0.24	0.20*	0.03; 0.38	0.14	-0.04; 0.31	0.16	-0.01; 0.33
	4	0.05	-0.19; 0.28	-0.13	-0.36; 0.10	-0.01	-0.25; 0.22	0.10	-0.09; 0.28	0.19	-0.00; 0.37	0.17	-0.02; 0.35
	5	0.12	-0.11; 0.34	-0.12	-0.34; 0.11	-0.00	-0.22; 0.22	0.20*	0.03; 0.38	0.14	-0.04; 0.32	0.16	-0.02; 0.33
Peripheral fat mass	0	-0.05	-0.17; 0.06	0.04	-0.08; 0.16	-0.02	-0.14; 0.09	0.13*	0.02; 0.24	0.07	-0.04; 0.18	0.14*	0.01; 0.24
	1	-0.05	-0.17; 0.07	0.04	-0.08; 0.16	-0.03	-0.15; 0.09	0.12*	0.01; 0.24	0.06	-0.06; 0.17	0.12*	0.01; 0.24
	2	-0.14	-0.35; 0.08	0.13	-0.09; 0.34	-0.04	-0.25; 0.18	-0.04	-0.20; 0.13	-0.01	-0.18; 0.16	0.06	-0.10; 0.22
	3	-0.13	-0.34; 0.09	0.13	-0.09; 0.34	-0.04	-0.25; 0.18	-0.03	-0.20; 0.13	-0.03	-0.19; 0.14	0.04	-0.12; 0.21
	4	-0.10	-0.31; 0.12	0.14	-0.08; 0.36	-0.03	-0.25; 0.19	0.04	-0.13; 0.21	-0.04	-0.21; 0.13	0.06	-0.11; 0.23
	5	-0.12	-0.34; 0.10	0.13	-0.08; 0.35	-0.03	-0.24; 0.19	-0.04	-0.20; 0.13	-0.00	-0.17; 0.16	0.07	-0.10; 0.23
Peripheral lean mass	0	-0.11	-0.23; 0.01	-0.02	-0.14; 0.09	-0.02	-0.14; 0.10	0.14*	0.03; 0.25	-0.02	-0.14; 0.07	-0.02	-0.14; 0.09
	1	-0.12	-0.24; 0.01	0.00	-0.12; 0.13	-0.02	-0.14; 0.10	0.13	0.01; 0.25	-0.05	-0.17; 0.07	-0.08	-0.19; 0.04
	2	-0.12	-0.24; 0.01	-0.00	-0.13; 0.12	-0.02	-0.14; 0.11	0.08	-0.05; 0.21	-0.09	-0.21; 0.04	-0.15*	-0.27; -0.02
	3	-0.11	-0.24; 0.01	-0.00	-0.13; 0.12	-0.02	-0.14; 0.11	0.08	-0.05; 0.20	-0.09	-0.21; 0.04	-0.14	-0.27; 0.02
	4	-0.11	-0.24; 0.01	-0.00	-0.13; 0.13	-0.02	-0.14; 0.11	0.06	-0.07; 0.18	-0.08	-0.21; 0.05	-0.15*	-0.27; -0.02
	5	-0.11	-0.23; 0.02	0.00	-0.13; 0.13	-0.01	-0.14; 0.12	0.08	-0.05; 0.20	-0.09	-0.22; 0.04	-0.14*	-0.27; -0.02
Anthropometrics													
Waist circumference	0	-0.08	-0.20; 0.04	0.03	-0.08; 0.15	0.01	-0.11; 0.13	0.20 [†]	0.09; 0.31	0.16 [†]	0.05; 0.27	0.15 [†]	0.04; 0.26
	1	-0.08	-0.20; 0.04	0.03	-0.09; 0.15	0.00	-0.12; 0.13	0.17 [†]	0.05; 0.29	0.14*	0.01; 0.26	0.14*	0.01; 0.26
	2	-0.08	-0.27; 0.12	0.10	-0.10; 0.29	0.17	-0.02; 0.37	0.10	-0.08; 0.28	0.19*	0.01; 0.37	0.08	-0.10; 0.25
	3	-0.08	-0.28; 0.11	0.10	-0.10; 0.29	0.17	-0.02; 0.37	0.10	-0.08; 0.28	0.20*	0.02; 0.38	0.08	-0.09; 0.26
	4	-0.15	-0.36; 0.05	0.09	-0.11; 0.29	0.17	-0.03; 0.37	0.04	-0.14; 0.22	0.21*	0.03; 0.39	0.07	-0.11; 0.26
	5	-0.08	-0.28; 0.11	0.09	-0.10; 0.29	0.17	-0.02; 0.37	0.10	-0.08; 0.28	0.19*	0.00; 0.37	0.06	-0.12; 0.24
Hip circumference	0	-0.07	-0.19; 0.05	-0.02	-0.13; 0.10	-0.08	-0.19; 0.04	0.18 [†]	0.07; 0.29	0.08	-0.03; 0.20	0.14*	0.03; 0.26

1	-0.07	-0.19; 0.06	-0.01	-0.13; 0.11	-0.08	-0.20; 0.04	0.16 [†]	0.04; 0.28	0.05	-0.07; 0.17	0.14*	0.01; 0.25
2	-0.01	-0.20; 0.19	-0.09	-0.28; 0.11	-0.22*	-0.41; -0.02	0.09	-0.08; 0.26	-0.08	-0.24; 0.09	0.08	-0.09; 0.25
3	-0.01	-0.20; 0.18	-0.08	-0.28; 0.11	-0.22*	-0.41; -0.02	0.11	-0.06; 0.27	-0.10	-0.27; 0.07	0.06	-0.11; 0.22
4	0.02	-0.17; 0.21	-0.08	-0.28; 0.11	-0.21*	-0.41; -0.20	0.11	-0.05; 0.28	-0.08	-0.24; 0.09	0.08	-0.09; 0.25
5	0.00	-0.19; 0.20	-0.08	-0.28; 0.11	-0.21*	-0.40; -0.02	0.09	-0.08; 0.26	-0.07	-0.24; 0.10	0.09	-0.08; 0.26

β, standardized regression coefficient; indicates the change in thrombin generation parameter (in SD) per 1 SD increase in determinant; CI, confidence interval

* $p < 0.05$, [†] $p < 0.01$, [‡] $p < 0.001$;

Model 0: crude;

Model 1: adjusted for age, prior CVD, glucose metabolism and smoking status;

Model 2: model 1 + the other body composition variables (DXA) or model 1+ either waist or hip circumference (anthropometrics);

Model 3: model 2 + SBP;

Model 4: model 2 + total cholesterol/HDL ratio, ln-triglycerides;

Model 5: model 2 + microalbuminuria

Figure 2. Associations between total body fat percentage and regional body composition, measured by DXA, and the thrombin generation parameters in women. Error bars represent 95% CI around association estimates. All data are adjusted for age, prior CVD, glucose metabolism, and smoking status. Right panel shows the (mutually adjusted) contributions of trunk fat, peripheral fat, and lean masses to these associations.

Waist and Hip Circumferences

The associations between fat distribution as estimated by means of waist and hip circumferences and study outcomes mimicked to a large extent the associations obtained with trunk and peripheral fat and lean masses as estimated by DXA (**Table 3**, model 1). After adjusting for hip circumference, the association between waist circumference and lag time and ETP in women was attenuated, whereas the association with peak height was stronger (model 2). Further adjustment for cardiovascular risk factors (models 3 to 5), if anything, only slightly attenuated these associations.

The anthropometric estimate of peripheral fat (ie, hip circumference) was positively associated with lag time and ETP in women (**Table 3**, model 1). These associations, however, disappeared after adjustment for waist circumference; and if anything, there was a trend toward a protective effect of hip circumference on peak height. Furthermore, there was a trend toward an inverse association between hip circumference and ETP in men (model 1), which became stronger after further adjustment waist circumference (model 2) and other cardiovascular risk factors (models 3 to 5).

Potential Mediation Role of Low-Grade Inflammation

The positive associations of estimates of total body fat (ie, total body fat percentage or BMI) and a central pattern of fat accumulation (ie, trunk fat mass or waist circumference) with the thrombin generation parameters observed in women were attenuated when further adjusted for hsCRP (**Table 4**).

Table 4. Mediation by low-grade inflammation in the association between total or central fat and thrombin generation in women

		Lag time			Peak height			ETP		
Determinant	Model	β	95%CI	'Mediating effect'*	β	95%CI	'Mediating effect'*	β	95%CI	'Mediating effect'*
DXA										
Total body fat %	1	0.13*	0.01; 0.25		0.13*	0.01; 0.25		0.21 [†]	0.10; 0.33	
	2	0.10	-0.03; 0.22	23%	0.08	-0.05; 0.20	38%	0.16*	0.04; 0.28	24%
Trunk fat mass	1	0.20 [†]	0.07; 0.32		0.11	-0.02; 0.23		0.17 [†]	0.04; 0.29	
	2	0.17*	0.04; 0.29	15%	0.05	-0.08; 0.18	55%	0.10	-0.03; 0.22	41%
Anthropometric s										
Body mass index	1	0.18 [†]	0.06; 0.30		0.12	-0.01; 0.24		0.16 [†]	0.04; 0.28	
	2	0.15*	0.03; 0.28	17%	0.07	-0.06; 0.19	42%	0.10	-0.02; 0.22	38%
Waist circumference	1	0.17 [†]	0.05; 0.29		0.14*	0.01; 0.26		0.14*	0.01; 0.26	
	2	0.14*	0.01; 0.27	18%	0.09	-0.04; 0.22	36%	0.07	-0.03; 0.20	50%

β , standardized regression coefficient; indicates the change in thrombin generation parameter (in SD) per 1 SD increase in determinant; CI, confidence interval

* Estimated as $(\beta_{\text{model 1}} - \beta_{\text{model 2}}) / \beta_{\text{model 1}} \times 100$;

[‡] $p < 0.05$, [‡] $p < 0.01$;

Model 1: adjusted for age, prior CVD, glucose metabolism and smoking status;

Model 2: model 1 + ln-hsCRP

Additional Analyses

Additional adjustment for use of platelet aggregation inhibitors or lipid-, blood pressure-, or glucose-lowering drugs did not materially affect any of the reported associations (data not shown). Excluding the few individuals who had an albumin-to-creatinine ratio above 30 mg/mmol (ie, macroalbuminuria, $n=5$) from the analyses also did not materially affect any of the results. Associations that were similar to those described above but slightly less strong were found when, instead, we used the thrombin generation parameters estimated in the presence of thrombomodulin (data not shown). Finally, we also investigated whether the associations between body composition and thrombin generation parameters differed across age categories by adding interaction terms into our models, but no such interactions were found (probability value for all, > 0.10).

Discussion

The present study had 3 major findings: first, total body fat mass was positively associated with a prolonged lag time, higher peak height, and higher ETP in women but not in men. Second, of the regional fat depots, trunk fat contributed the most to these associations, whereas there was no association with peripheral fat and a trend toward an inverse association with peripheral lean mass. Finally, the association between body fat and thrombin generation was independent of traditional cardiovascular risk factors and was to an important extent attenuated by low-grade inflammation.

Our findings are in line with a pilot study, performed in 13 extremely obese children, which reported a positive association between severe obesity (estimated by BMI) and lag time, peak height, and ETP (13), and with a recent study showing a significant, weight-loss-related decrease in these thrombin generation parameters in 36 morbidly obese adults 2 years after bariatric surgery (14). In addition, an association has been found between prothrombin 1.2 fragments and waist circumference in postmenopausal women (7). In the latter study, a trend towards an inverse association between hip circumference and prothrombin 1.2 fragments was described. Such an inverse association with hip circumference was also observed in our study, but more strongly so in men. This finding could reflect a protective role for either peripheral fat or lean mass. Our analyses with DXA estimates of regional body composition suggested that peripheral lean (but not fat) mass counteracted the adverse effects of central fat in this study.

The prolonged lag time in individuals with greater levels of body fat may be attributable to increased TF pathway inhibitor levels, which is one of the main determinants of lag time at the low TF concentrations used (24). Elevated TF pathway inhibitor levels have been

reported in individuals with impaired glucose tolerance and type 2 diabetes (25), and it has been suggested that this could be due to related obesity (26).

The reason why (central) body fatness was associated with thrombin generation only in women is unclear. Men and women differ with respect to total adiposity and fat distribution in that women store more fat in subcutaneous areas, especially in the gluteal and thigh regions, whereas men accumulate fat preferentially in the upper-body and visceral compartments. Effect modification by sex in the association between (central) fat and thrombin generation could be partially attributable to underlying hormonal differences between the sexes. For instance, sex-specific patterns in growth hormone secretion and aging-related changes in sex-steroid hormonal balance are associated with preferential accumulation of fat in the upper body (27), and both have been also associated with coagulation and thrombosis (28,29). In the present study, no such hormonal data were obtained, and therefore we cannot further determine their potential role. Other obesity-related sex-specific factors may, however, play a role as well. Several studies have found that (older) women have higher levels of low-grade inflammation than men with comparable levels of obesity and that the association between central obesity and hsCRP is stronger in women than men (30,31). Indeed, in the present study, the association between central fat mass and hsCRP was also stronger in women than in men (age, prior CVD, glucose metabolism, and smoking status adjusted standardized β_{women} : 0.33 [95%CI, 0.22 to 0.44] versus β_{men} : 0.23 [0.12 to 0.34]; probability value for interaction, < 0.10), and indeed, the associations between central fatness and thrombin generation in women were, to an important extent (up to 55%), attenuated by low-grade inflammation. Nevertheless, and albeit statistically significant, differences between men and women as reported in this study, as well as their underlying biological mechanisms, if any, need to be examined in future studies.

Previous cross-sectional studies have shown positive associations between hsCRP and prothrombin 1.2 fragments in individuals with and without prior CVD (32,33). One proposed mechanism by which hsCRP, or low-grade inflammation in general, may lead to increased thrombin generation may be by inducing endothelial dysfunction, which in turn leads to increased TF secretion by monocytes (34), a phenomenon observed only in the presence of and through direct interaction with other leukocytes (35). On the basis of this evidence and also on the well-established association between (central) obesity and low-grade inflammation (15), we hypothesized that low-grade inflammation could mediate the positive associations between (central) fatness and thrombin generation. Our findings indeed seemed to support this hypothesis. However, the relationship between inflammation and coagulation may be bidirectional, as activated coagulation proteases

may affect specific cellular receptors on inflammatory and endothelial cells, thereby modulating an inflammatory response (36). When we reanalyzed our data under this alternative hypothesis, we found no appreciable “mediation effects” of thrombin generation in the associations between (central) obesity and low-grade inflammation (hsCRP) (data not shown). An alternative hypothesis that we cannot discard on the basis of the design of the present study is that inflammation and thrombin generation are both elevated as a consequence of (central) obesity without being causally related to each other. Additional mechanistic studies investigating the cross-talk, if any, between hsCRP but also other proinflammatory adipose tissue-related factors (such as interleukin-6, intercellular adhesion molecule, serum amyloid A, and leptin) and thrombin generation, and the extent to which any such associations differ between sexes, are thus warranted.

Not only an increased procoagulant activity but also a decreased anticoagulant activity may lead to higher thrombin generation. When thrombin generation is measured in the presence and absence of thrombomodulin, an estimate of the function of the anticoagulant protein C system is obtained. In our study, no association was found between body composition and the thrombomodulin ratio. This indicates that body fat and its distribution are not associated with abnormalities in protein C activation, which is in line with a previous study in individuals with morbid obesity (37).

Strengths of our study were the assessment of both anthropometric and more precise estimates of body composition (DXA) and the investigation of how both types of estimates of body composition related to thrombin generation. We found that the strength of the associations between BMI and waist circumference or total body fat percentage and its distribution, measured by DXA, on the one hand and thrombin generation on the other were similar. This suggests that in large epidemiological studies on obesity-related hypercoagulability, anthropometric estimates of obesity/fat distribution might be a reliable alternative to DXA or other objective estimates of body composition when these are unavailable. In addition, the body composition–thrombin generation associations were investigated in a relatively large population-based group of extensively described individuals (17–19), which allowed adjustment for many other cardiovascular risk factors, such as hypertension, dyslipidemia, smoking, and microalbuminuria. Finally, the CAT method we used to estimate thrombin generation mimics the overall potential to clot whenever a thrombogenic stimulus (eg, TF release after plaque rupture) appears. In theory, this method may give a more reliable estimation of coagulation under pathophysiological conditions than the single-point measurement of activation markers, such as prothrombin 1.2 fragments or thrombin-antithrombin complexes (38). However,

prospective studies comparing the value of these measures in the prediction of prothrombotic events are needed to further clarify this issue.

There are some limitations to our study. First, because of its cross-sectional design, any inferences about causality should be made with caution. Second, because fat measures are strongly related to each other, it can be argued that adjustment of these measurements for each other (eg, **Table 3**) was not adequate, leading to overcorrected models. However, although the regression coefficients changed considerably after mutual adjustments for other body composition variables, our analyses were not disturbed by multicollinearity (ie, tolerance was > 0.1 in all models). In fact, these analyses allowed us to tease apart the relative contributions of different fat depots and lean mass in the associations examined. Still, the clinical relevance of the obesity-related elevations in thrombin generation observed in the present study needs to be clarified, as the strength of the associations we found were, albeit significant, relatively weak (and body fatness explained only up to 5% of the variance in the parameters investigated). Third, despite the fact that trunk fat as assessed by DXA correlates highly with intraabdominal fat, it does not distinguish between subcutaneous and visceral fat in the trunk/abdominal region (39). Whether the associations of thrombin generation with visceral fat differ from those with (abdominal) subcutaneous fat need to be further examined. Finally, we studied a white, elderly population, and it remains to be established whether the results can be generalized to other ethnicities and to younger individuals.

In conclusion, we have shown that, in women, total body fat is positively associated with thrombin generation in plasma. Of the regional fat depots, central fat mass was the strongest contributor to this association, which may be explained, at least in part, by central-obesity related low-grade inflammation. Prospective and mechanistic studies are needed to further clarify the mechanisms underlying the obesity-thrombin generation relationships and to ascertain the extent to which higher thrombin generation explains the excess of atherothrombotic events associated with obesity.

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Disclosures

None

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Chapter 7

Impaired glucose metabolism and type 2 diabetes are associated with hypercoagulability: potential role of central adiposity and low-grade inflammation. The Hoorn Study.

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Abstract

Introduction — Type 2 diabetes (DM2) is associated with greater risk for cardiovascular disease (CVD), which may, at least partially, be explained by prothrombotic alterations. We therefore investigated; first, the extent to which individuals with impaired glucose metabolism (IGM) and/or DM2 had greater levels of thrombin generation than those with normal glucose metabolism (NGM); and second, whether any differences were independent of other cardiovascular risk factors, such as smoking, hypertension, dyslipidaemia, (micro)albuminuria, glycemic control and (central) adiposity, and/or were potentially 'mediated' by low-grade inflammation (high-sensitivity C-reactive protein (hsCRP)).

Materials and Methods— We studied 744 individuals from the Hoorn Study (275 NGM, 176 IGM and 293 DM2, mean age 68.6 ± 7.1 years). Thrombin generation in platelet-poor plasma was measured using the Calibrated Automated Thrombogram and three parameters were derived: lag time, peak height and endogenous thrombin potential (ETP). Data were analyzed with multiple linear regression analyses.

Results — After adjustment for age, sex, prior CVD and smoking status, individuals with IGM or DM2 had a longer lag time [$\beta = 0.14$ min (95% CI: 0.02; 0.26)], higher peak height [$\beta = 7.29$ nM (-1.33; 15.91)] and ETP [$\beta = 35.65$ nM*min (0.97; 70.34)] than those with NGM. These differences were attenuated to $\beta = 0.06$ min (-0.07; 0.19), 3.82 nM (-5.46; 13.10) and 16.34 nM*min (-20.92; 53.59), respectively, when further adjusted for waist circumference and hsCRP.

Conclusion — Individuals with IGM or DM2 had up to 4% higher thrombin generation compared with NGM, which may be explained, to a great extent, by the greater levels of central adiposity and related low-grade inflammation characterizing these individuals.

Introduction

Individuals with type 2 diabetes (DM2) have a 2- to 3-fold higher risk for cardiovascular disease (CVD) as compared with non-diabetic individuals (1). A substantial portion of diabetes-related macrovascular complications is due to atherothrombotic events (2) which could, at least in part, be explained by an association between impaired glucose metabolism and thrombin generation. Indeed, thrombin plays a central role in the coagulation cascade (3,4) and elevated levels of thrombin-antithrombin (TAT) complexes and prothrombin 1.2 fragments (both *in vivo* markers of thrombin generation) have been reported in patients with DM2 (5–7).

Recently, several new methods have become available which quantitatively measure thrombin generation, *in vitro*, after activation of the coagulation cascade with tissue factor (TF), phospholipids and calciumchloride (CaCl_2) (8). The Calibrated Automated Thrombogram (CAT) is one such method (9,10). Specifically, it generates a thrombin generation curve that estimates the overall plasma coagulability potential. The endogenous thrombin potential (ETP), or the area under this curve, represents the total amount of active thrombin that can be formed after activation of the coagulation cascade. Measured as such, the ETP has been shown to be a good indicator of a hypercoagulable state in, for example, individuals using oral contraceptives, or who had prior venous thromboembolism (9,11–13). Because the CAT method mimics the overall potential to clot whenever a thrombogenic stimulus (e.g. TF release after plaque rupture) appears, it is thought to provide a more reliable estimation of coagulation under pathophysiological conditions than the single point measurement of activation markers such as prothrombin 1.2 fragments or TAT complexes (14).

Only one small case-control study has investigated the association between DM2 and thrombin generation according to this method (15). However, no studies have been performed at the population level. Establishing such an association is a necessary step to ascertain its potential value as a tool to estimate atherothrombotic risk in individuals with impaired glucose metabolism (IGM) and/or DM2. Furthermore, from an etiological point of view, it is not clear whether any such association is independent of other risk factors that typically characterize these individuals (e.g. hypertension and central adiposity) or, alternatively, is explained by these risk factors and their associated pathophysiological mechanisms. Central adiposity and related low-grade inflammation are likely candidates in this regard (16). Indeed, individuals with IGM and DM2 have higher levels of high-sensitivity C-reactive protein (hsCRP) which are, to a great extent, attributable to their

higher levels of (central) adiposity (17). In addition, in vivo, hsCRP has been associated with increased thrombin generation (assessed by activation markers) (18).

In view of these considerations, we investigated, in a population-based cohort, first, whether individuals with IGM and/or DM2 had greater levels of thrombin generation in plasma than those with NGM; and second, whether any such differences were independent of other cardiovascular risk factors and/or explained by (adiposity-related) low-grade inflammation (hsCRP).

Materials and methods

Study population

For the present investigation, we used data from the 2000 Hoorn Study follow-up examination and, to increase the number of individuals with DM-2, data from The Hoorn Diabetes Screening Study, both of which were population-based and have been described in detail elsewhere (16,19,20). Briefly, the Hoorn Study is a cohort study of glucose metabolism in the general population (n=2484), which started in 1989 (20). In 2000, a follow-up examination was carried out among all surviving participants who had given their permission to be recontacted. We invited all those who had diabetes, as determined by an oral glucose tolerance test, or who were treated for diabetes at the previous 1996 follow-up (n=176). Next, we invited random samples of individuals with normal glucose metabolism (NGM) (n=705) and IGM (n=193). Of 1074 individuals thus invited, 648 (60%) participated [19]. Additionally, we invited 217 individuals with DM-2 from the Hoorn Screening Study (21), of whom 188 (87%) participated. All participants underwent a glucose tolerance test, except those with previously diagnosed diabetes (n=67). Data on 14 individuals were missing because of logistical problems. Glucose tolerance was defined according to the 1999 WHO criteria (22). Altogether, the eligible cohort thus consisted of 822 individuals. For the present study, participants using cumarin derivatives (n=25) and/or hormonal replacement therapy (n=3) and/or having missing data on thrombin generation parameters (n=54) and/or in whom glucose metabolism status could not be ascertained (n=14) were excluded. The present study therefore consisted of 744 individuals: 275 with normal glucose metabolism (NGM), 176 with IGM (including those with impaired fasting glucose and/or impaired glucose tolerance) and 293 with DM2.

The Ethical Review Committee of the VU University Medical Centre approved the study protocol and all participants gave their written informed consent.

Thrombin generation parameters

Venous blood samples were collected in 2000–2001 and 3.2% citrated platelet-poor plasmas (PPP) were prepared by two-step centrifugation: at 4000 $\times g$ for 15 minutes followed by centrifugation at 10000 $\times g$ for 5 minutes. Plasmas were stored at -80°C until analysis in 2008 and had not been thawed before. Thrombin generation in PPP was measured using the CAT-method (10), which employs low-affinity fluorogenic substrate for thrombin (Z-Gly-Gly-Arg-AMC) to continuously monitor thrombin activity in clotting plasma. Thrombin generation was assessed according to manufacturer's instructions (Thrombinoscope BV) in a 96-well plate fluorometer (Ascent Reader, Thermolabsystems OY, Helsinki, Finland) as previously described (23). Briefly, thrombin generation was determined in the presence of 1 pM TF and 4 μM phospholipids in the absence and presence of 0.55 nM recombinant soluble thrombomodulin (Paion GmbH, Aachen, Germany). The 1 pM TF trigger was a commercial product (PPP Reagent Low; Thrombinoscope BV, Maastricht, the Netherlands). Three parameters were derived from these analyses, i.e. lag time (min), peak height (nM) and ETP (nM $\cdot$ min). A thrombomodulin ratio was calculated by dividing the ETP and peak height in the presence by those in the absence of thrombomodulin. This ratio indicates the percentage inhibition of thrombin generation by activation of the anti-coagulant protein C pathway. At our lab, the intra-assay coefficients of variation (CVs) are $<5\%$ (13) and the inter-assay CVs, obtained in 2 lots of normal-pool plasma in 25 independent runs during the present study, were $<11\%$ for all thrombin generation parameters (calculated using the EP Evaluator 8.0.0.90 software).

Other measurements

Health status, medical history, medication use and smoking habits were assessed by questionnaires (20). We measured waist circumference, body mass index (BMI), systolic (SBP) and diastolic blood pressure (DBP), levels plasma glucose (fasting and post-load), HbA1c, total, high-(HDL) and low-density lipoprotein (LDL) cholesterol, triglycerides, urinary albumin-to-creatinine ratio and hsCRP as described in detail elsewhere (20,24,25). Hypertension was defined as SBP ≥ 140 mmHg and/or DBP ≥ 90 mmHg and/or the use of anti-hypertensive drugs (26). Participants were classified as having microalbuminuria if the albumin-to-creatinine ratio was ≥ 2.0 mg/mmol (24). Prior CVD was defined as described previously (16).

Statistical analyses

Variables with a skewed distribution (i.e. triglycerides and hsCRP) were log-transformed prior to further analyses. Comparisons of the clinical characteristics according to

individuals' glucose metabolism status were investigated by means of analyses of variance (ANOVA) for continuous variables and chi-squared tests for frequency data.

We used multiple linear regression analyses to investigate the differences in thrombin generation parameters between individuals with IGM or DM2 vs. NGM (reference group). These analyses were first adjusted for age, sex, prior CVD and smoking status (model 1), and additionally for measures of blood pressure, dyslipidemia, (micro)albuminuria, glycemic control, (central) adiposity and low-grade inflammation (models 2 to 7). In addition, we investigated whether the attenuation by low-grade inflammation was adiposity driven by adding adjustments for both waist circumference and hsCRP (model 8). In all these analyses individuals with IGM and DM2 were combined into one group, since the parameters of thrombin generation examined herein did not substantially differ between these two groups. In all analyses described above, dependent variables were the thrombin generation parameters obtained in both the absence and presence of thrombomodulin.

Statistical significance was set at a p-value of <0.05. All analyses were performed with the use of the Statistical Package for Social Sciences (SPSS) for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA).

Results

Participants who were excluded from the analyses (n=78) were younger, more often obese and more often had diabetes than the ones included (data not shown). The clinical characteristics of the participants included (n=744) are shown in **Table 1** according to their glucose metabolism status. A deteriorating glucose metabolism status was associated with a larger waist circumference and higher levels of dyslipidemia, blood pressure, microalbuminuria, hsCRP and prior CVD. In addition, participants with IGM and DM2 more often used lipid-lowering drugs and platelet aggregation inhibitors than those with NGM.

Levels of lag time, peak height and ETP assessed in the absence of thrombomodulin were comparable in individuals with IGM and DM2 and greater in these individuals than in those with NGM (**Table 2, Figure 1**). Activating the anticoagulant protein C pathway by adding thrombomodulin did not affect the absolute values of lag time but led to lower absolute values of peak height and ETP, which were reduced by approximately 14 and 35%, respectively, in all groups (**Table 2**). Despite the relatively higher values in individuals with IGM and DM2 under these conditions, peak height and ETP did not differ significantly from those of individuals with NGM, however lag time remained significantly prolonged.

The thrombomodulin ratio of peak height and ETP did not materially differ between the 3 groups (**Table 2**).

Table 1: Clinical characteristics according to glucose metabolism status

	NGM (n=275)	IGM (n=176)	DM2 (n=293)	<i>p</i> for linear trend
Age (years)	68.5 ± 6.0	70.3 ± 6.5	67.6 ± 8.0	0.099
Sex (% male)	47.3	50.6	50.9	0.397
Fasting glucose (mmol/l)	5.42 ± 0.37	6.08 ± 0.48	7.71 ± 1.80	<0.001
Glycated haemoglobin (%)	5.68 ± 0.41	5.88 ± 0.39	6.61 ± 0.92	<0.001
Body mass index (kg/m ²)	26.2 ± 3.3	27.8 ± 4.1	29.1 ± 4.6	<0.001
Waist circumference (mm)	90.7 ± 11.0	97.3 ± 10.8	101.1 ± 12.0	<0.001
Total cholesterol (mmol/l)	5.79 ± 1.03	5.79 ± 1.02	5.53 ± 1.06	0.003
LDL-cholesterol (mmol/l)	3.71 ± 0.91	3.68 ± 0.92	3.46 ± 0.92	0.001
HDL-cholesterol (mmol/l)	1.51 ± 0.41	1.44 ± 0.40	1.24 ± 0.34	<0.001
Total-to-HDL cholesterol ratio	4.05 ± 1.09	4.32 ± 1.39	4.71 ± 1.32	<0.001
Triglycerides (mmol/l)	1.20 [0.90-1.50]	1.30 [1.00-1.70]	1.60 [1.20-2.20]	<0.001
Lipid lowering drugs (%)	12.4	15.9	19.9	0.016
Systolic blood pressure (mmHg)	137 ± 20	143 ± 18	146 ± 21	<0.001
Diastolic blood pressure (mmHg)	81 ± 11	83 ± 10	85 ± 10	<0.001
Mean arterial pressure (mmHg)	95 ± 11	100 ± 10	102 ± 11	<0.001
Use of antihypertensives (%)	23.4	37.5	50.3	<0.001
Microalbuminuria (%)	9.8	15.9	18.8	0.003
Smoking (%)	13.9	17.6	12.1	0.525
Prior CVD (%)	45.1	50.0	53.2	0.053
Use of Platelet aggregation inhibitors (%)	13.2	16.5	19.8	0.038
High-sensitivity C-reactive protein (mg/l)	1.52 [0.80; 3.14]	2.19 [1.29; 4.61]	3.14 [1.42; 7.00]	<0.001

Results are expressed as percentages (%), mean ± SD, or median [inter-quartile range]

NGM: normal glucose metabolism; IGM: impaired glucose metabolism; DM2: type 2 diabetes mellitus; LDL: low density lipoprotein; HDL: high density lipoprotein; CVD: cardiovascular disease

Figure 1. Thrombin generation curves according to glucose metabolism status i.e. normal glucose metabolism (NGM), impaired glucose metabolism (IGM) or type 2 diabetes mellitus (DM2).

Table 2. Thrombin generation parameters according to glucose metabolism status

	NGM (n=275)	IGM (n=176)	DM2 (n=293)	p-value (IGM + DM2 vs. NGM)
<u>Analyses without thrombomodulin</u>				
Lag time (min)	3.98 ± 0.83	4.10 ± 0.80	4.14 ± 0.77	0.021
Peak Height (nM)	182 ± 57	191 ± 58	189 ± 59	0.067
ETP (nM*min)	1166 ± 222	1218 ± 237	1192 ± 238	0.034
<u>Analyses with thrombomodulin</u>				
Lag time (min)	3.91 ± 0.68	4.06 ± 0.71	4.06 ± 0.76	0.005
Peak height (nM)	159 ± 60	166 ± 60	167 ± 65	0.104
ETP (nM*min)	776 ± 253	810 ± 256	807 ± 261	0.106
<u>Thrombomodulin ratio</u>				
TM ratio Peak height n=737	0.86 ± 0.11	0.86 ± 0.10	0.86 ± 0.14	0.867
TM ratio ETP n=737	0.65 ± 0.14	0.65 ± 0.14	0.66 ± 0.15	0.559

Results are expressed as mean ± SD

NGM: normal glucose metabolism; IGM: impaired glucose metabolism; DM2: type 2 diabetes mellitus; ETP endogenous thrombin potential; TM: thrombomodulin

Additional analyses

Additional adjustment for use of platelet aggregation inhibitors, or lipid- , blood pressure- or glucose-lowering drugs did not materially affect any of the reported associations (data not shown). We also investigated whether the association between glucose metabolism status and thrombin generation parameters differed between men and women or between different age categories, by adding interaction terms to our models. However, no such interactions were found (p-values for interaction all > 0.10).

Table 3. Thrombin generation parameters according to glucose metabolism status

Model	Adjustments	Lag time (min)		Peak height (nM)		ETP (nM*min)	
		β	95%CI	β	95%CI	β	95%CI
Without thrombomodulin							
0	Crude	0.14*	0.02; 0.26	7.98	-0.63; 16.58	36.75*	2.17; 71.34
1	Age, sex, prior CVD, smoking	0.14*	0.02; 0.26	7.29	-1.33; 15.91	35.65*	0.97; 70.34
2	Model 1 + SBP	0.13*	0.01; 0.25	6.42	-2.34; 15.18	32.80	-2.46; 68.07
3	Model 1 + total cholesterol/ HDL ratio and ln-triglycerides	0.04	-0.08; 0.16	8.16	-0.79; 17.11	34.29	-1.88; 70.47
4	Model 1 + microalbuminuria	0.12*	0.01; 0.24	7.10	-1.57; 15.76	34.31	-0.56; 69.17
5	Model 1 + HbA1c	0.07	-0.06; 0.20	6.95	-2.49; 16.39	41.54*	3.56; 79.52
6	Model 1 + waist circumference	0.09	-0.04; 0.22	5.48	-3.76; 14.71	23.85	-13.26; 60.97
7	Model 1 + ln-hsCRP	0.09	-0.03; 0.21	4.57	-4.22; 13.36	22.90	-12.40; 58.19
8	Model 1 + waist circumference and ln-hsCRP	0.06	-0.07; 0.19	3.82	-5.46; 13.10	16.34	-20.92; 53.59
With thrombomodulin							
0	Crude	0.15 [†]	0.04; 0.26	7.54	-1.66; 16.74	31.21	-6.90; 69.33
1	Age, sex, prior CVD, smoking	0.15 [†]	0.04; 0.25	6.80	-2.41; 16.02	27.89	-10.28; 66.03
2	Model 1 + MAP	0.13*	0.03; 0.24	6.18	-3.19; 15.55	26.39	-12.42; 65.21
3	Model 1 + total cholesterol/ HDL ratio and ln-triglycerides	0.07	-0.04; 0.18	7.95	-1.62; 17.53	34.87	-4.86; 74.60
4	Model 1 + microalbuminuria	0.13*	0.02; 0.23	6.78	-2.48; 16.04	27.42	-10.95; 65.79
5	Model 1 + HbA1c	0.08	-0.03; 0.20	6.60	-3.49; 16.69	32.42	-9.37; 74.21
6	Model 1 + waist circumference	0.09	-0.03; 0.20	5.15	-4.72; 15.02	22.07	-18.83; 62.97
7	Model 1 + ln-hsCRP	0.09	-0.02; 0.20	4.26	-5.15; 13.66	17.15	-21.80; 56.10
8	Model 1 + waist circumference and ln-hsCRP	0.06	-0.06; 0.17	3.60	-6.34; 13.53	15.43	-25.71; 56.56

Regression coefficient (β) with respective 95%CI indicates the difference in thrombin generation parameter (in respective units) in IGM/DM2 vs. NGM. * $p < 0.05$, [†] $p < 0.01$

Discussion

The main findings of this study were two-fold: first, individuals with IGM and DM2 had comparable levels and both had higher levels of thrombin generation than those with NGM, but the magnitude of these differences was relatively small (up to 4%). Second, central adiposity and related low-grade inflammation explained to a great extent the association between glucose tolerance status and thrombin generation, i.e., peak height and ETP.

The modest elevations in thrombin generation parameters suggest that thrombin generation in PPP only slightly contributes to the hypercoagulable state in IGM and DM2. This observation is in line with a recently published case-control study in DM2 patients that also reported minimal differences between DM2 patients compared with healthy controls (15). Alterations in primary hemostasis and/or the fibrinolytic system may thus be more important in establishing the hypercoagulable state in IGM and/or DM2 than those

reflected by the thrombin generation assay on which we focused herein. For example, DM2 is associated with increased platelet activation and aggregation and von Willebrand factor levels (27,28) and plasminogen activator inhibitor (PAI)-1 levels are elevated in DM2, probably due to an increased hepatic synthesis (29). PAI-1 inhibits fibrinolysis, which is associated with more resistant clot formation and possibly plays a role in vascular complications such as atherothrombosis.

The thrombin generation parameters were elevated to a similar extent in individuals with IGM and DM2, which is in line with a previous study (30). These findings support the 'ticking clock hypothesis' suggesting that hemostatic disturbances may be present even before fully developed diabetes (31). The prolonged lag time in IGM and DM2 may be explained by elevated tissue factor pathway inhibitor (TFPI) levels, which is one of the main determinants of lag time at the low TF concentrations used in our study (23). In addition, previous studies have shown a positive association between glucose metabolism status and TFPI (30,32). In the present study, the association between glucose metabolism status and lag time was partially attenuated by dyslipidemia, which is in line with previous studies that have shown a positive association between dyslipidemia and TFPI (33,34).

The associations between glucose metabolism status and peak height and ETP were to a great extent attenuated by waist circumference, suggesting that central adiposity may be a common antecedent of both IGM/DM2 and increased thrombin generation. We have previously shown in this cohort that individuals with a central pattern of fat distribution indeed had higher levels of thrombin generation in plasma, which were to a great extent explained by adiposity-related increases in hsCRP (16). Furthermore, a recent meta-analysis supports these findings by suggesting that the elevated hsCRP levels in DM2 are probably due to central adiposity (17). One proposed mechanism by which hsCRP, or low-grade inflammation in general, may lead to increased thrombin generation may be by inducing endothelial dysfunction which in turn leads to increased TF expression by monocytes (35), a phenomenon which is only observed in the presence of and through direct interaction with other leukocytes (36). Taken together, our findings thus suggest that adiposity-related low-grade inflammation and not hyperglycemia per se might be one of the main driving factors behind the association between glucose metabolism status and thrombin generation in PPP. In addition, previous studies have not found an association between glycemic control and prothrombin 1.2 fragments and/or TAT-complexes (37,38). However, these studies did find an association between glycemic control and thrombin generation in platelet-rich plasma, suggesting that associations between glycemic control and thrombin generation might mainly be a platelet-dependent phenomenon (39).

There are some limitations to our study that need to be mentioned. First, due to its cross-sectional design, any inferences about causality should be made with caution. Second, as we studied a relatively elderly population, we cannot fully exclude the possibility that selective mortality in the diabetes group (i.e. many 'healthy' survivors included in this study) may have explained their comparable (instead of higher) levels of thrombin generation parameters as compared with the individuals with IGM. Third, we studied a Caucasian, elderly population and it remains to be established whether these results can be generalized to other ethnicities and/or to younger individuals. Fourth, measures of thrombin generation were performed in plasma samples that were stored for 7 to 8 years. Although in our lab we have been able to rule out effects of approximately one year of storage time on ex vivo thrombin generation, long term effects, if any, are largely unknown. If long term storage introduced a systematic error in measures of thrombin generation, we can rule out its influence on the differences found between the groups examined in the present study, since storage time was similar for all plasma aliquots. If random errors were introduced, or even if storage time reduced the variation of the measures as captured by our assay, then the differences between groups reported herein were most likely underestimated. Finally, it should be noted that, in the present cohort, it is unknown which individuals carried the factor V Leiden mutation. In general, these individuals will have a higher thrombin generation due to activated protein C resistance and an association between DM2 and the factor V Leiden mutation has been previously reported (40). However, several other studies, including a Dutch one, did not find any association between DM2 and factor V Leiden mutation (41–43). So far, no studies have investigated the association between DM2 and acquired activated protein C resistance. We have shown herein that activating the protein C pathway by adding thrombomodulin did not materially change the strength of the associations between glucose metabolism status and peak height or ETP, which indicates that there were no abnormalities in protein C activation in individuals with IGM or DM2.

In conclusion, we have shown that IGM and DM2 are associated with higher thrombin generation in PPP, which can be explained, to a large extent, by their greater levels of central adiposity and related low-grade inflammation. It remains to be established whether the assessment of thrombin generation in PPP constitutes a useful method for the characterization of hypercoagulability, relevant to arterial and venous thrombosis, in these individuals, since the magnitude of the differences was relatively small.

Conflict of interest statement

None

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Chapter 8

Discussion and Summary

Discussion and Summary

The main objective of this thesis was to increase current knowledge about the underlying mechanisms potentially explaining the increased cardiovascular disease (CVD) risk in individuals with central obesity, the metabolic syndrome (MetS) or type 2 diabetes (DM2). We investigated associations with intermediate phenotypes of CVD such as endothelial dysfunction, microalbuminuria, cardiovascular autonomic dysfunction, arterial remodeling and hypercoagulability. We used data from the Hoorn study, a population-based cohort study on glucose metabolism and its complications in elderly individuals, and the Amsterdam Growth and Health Longitudinal Study (AGAHLS), a longitudinal study on the life-course relationships between biological and lifestyle risk factors in young adults.

In this final chapter we summarize the main findings, address some methodological considerations and discuss potential (clinical) implications and directions for future research.

MAIN FINDINGS

In **Chapter 2** we investigated, in elderly individuals without and with type 2 diabetes, the association between body composition and endothelial (dys)function defined on the basis of brachial flow-mediated dilation (FMD) and circulating biomarkers. We observed a positive, i.e. beneficial, association between peripheral lean mass and FMD, and a negative, i.e. adverse, association between (central) fat mass and circulating biomarkers of endothelial function. These findings suggest that not only central fatness, but also sarcopenia may independently contribute to endothelial dysfunction in elderly individuals, possibly through differential effects on specific aspects of this multifaceted construct.

In **Chapter 3** we showed, again in elderly individuals without and with type 2 diabetes, that microalbuminuria and cardiovascular autonomic dysfunction were associated with cardiovascular mortality independently of each other, which suggests that they may be linked with cardiovascular mortality through different biological pathways.

In **Chapters 4 and 5** the associations between MetS and structural adaptations of the carotid artery were studied in a cross-sectional (**Chapter 4**) and a longitudinal (**Chapter 5**) setting in elderly and young individuals, respectively. We showed that MetS was cross-sectionally associated with arterial properties consistent with a pattern of maladaptive remodeling of the carotid artery in elderly individuals (**Chapter 4**). In addition, we showed

that persistent MetS over the course of 6 years was also associated with this adverse pattern of arterial remodeling in young adults and that recovery from MetS was associated with restoration of the arterial properties to levels comparable to the individuals who remained without MetS, emphasizing the potential for reversibility (**Chapter 5**).

Finally, **Chapter 6** demonstrated that body fat mass, and in particular a central pattern of fat distribution, was associated with higher levels of thrombin generation in elderly women, but not in men. This association was to a great extent attenuated by adiposity-related low-grade inflammation. In addition, we showed that individuals with impaired glucose metabolism (IGM) and DM2 had comparable levels of thrombin generation, both significantly higher than those with normal glucose metabolism, and that this difference was explained, to a great extent, by their higher levels of adiposity-related low-grade inflammation (**Chapter 7**).

METHODOLOGICAL CONSIDERATIONS

Study populations and designs – external validity

Most of the studies included in this thesis (all except **Chapter 5**) were conducted within the Hoorn study, and consisted of (a selection of) a Caucasian, elderly population with a relatively high risk of CVD. Whether our results can be extrapolated to other ethnicities and/or younger/healthier individuals is unknown and should be investigated in future studies.

The extrapolation of the results obtained in the study conducted within the AGAHLs (**Chapter 5**) may also be limited because this cohort consists of individuals with relatively high education and socio-economic levels and of mostly (>95%) Caucasian ethnicity. The prevalence of main risk factors, such as those comprised in the MetS may have been lower than in the general Dutch population, as these risk factors tend to be higher among subjects with lower socio-economic status and ethnic minorities.

However, issues around the representativeness of the cohorts used in the present study are not likely to affect the pathophysiological validity of our findings.

Study populations and designs – internal validity

Potential sources of bias that may obscure the internal validity and thus inferences of ‘cause and effect’ drawn from observational studies are: *selection bias*, *confounding* and *information bias*.

Selection bias. As mentioned above, for most studies included in this thesis we used data from the Hoorn Study, a population-based cohort study on glucose metabolism and its complications that started in 1989. However, our studies – specifically those in **Chapters 2,4,6 and 7** - were based on cross-sectional data from the follow-up examination carried out in 2000-2001, when the majority of the intermediate phenotypes of interest (i.e. arterial remodeling, thrombin generation, comprehensive set of circulating biomarkers of endothelial dysfunction and low-grade inflammation) were measured for the first time. Selective attrition could thus have introduced some bias into the estimates reported in these studies because individuals who participated in the follow-up examination were relatively healthier than the non-responders or those who died. Therefore, most likely our estimates reflected an underestimation of the true associations, because risk factors such as (central) obesity, MetS and/or DM2 (the main determinants of interest in our studies) and maladaptive remodeling, thrombin generation and low-grade inflammation and endothelial dysfunction (the main outcomes) are known determinants of mortality (**Figure 1**).

The results obtained in the AGAHLs (**Chapter 5**) were not likely affected by selection bias. Although the sample used (n=373 at the age of 36 and 297 of these who had also arterial data at the age of 42) was considerably reduced in size as compared with the original cohort at baseline (n=698 adolescences), it did not differ from the drop-outs with regard to body fatness and other biological risk factors at the beginning of the study (in 1976) (3, 4). The young age of the cohort probably has prevented the occurrence of selective drop-out due to (chronic) illness and/or mortality, with drop-outs thus far (often intermittent, i.e. at one wave but returning at a subsequent one) being most likely due to the quite long follow-up duration of the study (30 years!).

Figure 1. Directed acyclic graph (DAG) depicting general causal structure underlying attrition-related selection bias in chapters 2,4,6,7 (1, 2). In this DAG, the risk factors of interest directly negatively influence (-) post-enrolment survival or continuation in the Hoorn study, which is a collider on the path between risk factor and outcome (indicated by the box). The outcomes, (current) maladaptive remodeling, thrombin generation, low-grade inflammation and/or endothelial dysfunction reflect (+) the levels of these parameters prior to study drop-out. On the other hand, these parameters likely negatively influenced (-) survival or study continuation. Analyses restricted to the participants who survived and continued in the study are a form of conditioning (on survival/participation) which, in this case (see signs depicting the direction between associations) can have induced a downward bias in the associations reported, which results in an underestimation of the effect sizes.

Confounding. Our results could have been affected by residual confounding, that is, a variable or variables associated with both the main determinant and the outcome of interest, without being in the causal pathway between the two, but that were not measured or taken into account in the analyses could have been responsible for the associations we reported. Both the Hoorn study and the AGAHLs participants were well characterized and an abundant set of potential confounders was measured. All analyses were therefore carefully adjusted for potential confounding factors relevant for the associations under investigation, as selected based on prior (aetiological) knowledge. However, we cannot fully exclude the possibility that unknown/unmeasured confounders may still have led to a distortion of the results (either over- or underestimation). Such residual confounding is difficult to solve in observational studies.

Information bias. Errors in measurements may have lead to misclassification of subjects (either differential or non-differential). Differential misclassification occurs when the errors differ across groups or levels of risk factor exposure. The effects of such misclassification can vary from an overestimation to an underestimation of the true associations (5). Non-differential misclassification occurs when the probability of being misclassified is equal for all groups/levels of risk factor exposure. In general, this may lead to an underestimation of the true association due to reduced power (6). Below, we discuss in more detail the issue of misclassification with regard to the measurements of the

specific determinants (i.e. body composition, MetS, DM2) and outcome variables (i.e. endothelial dysfunction, cardiovascular autonomic dysfunction, remodeling of the carotid artery and thrombin generation) of interest in this thesis.

In addition to the aspects of external and internal validity discussed above, it should be noted that inferences of cause and effect drawn from the studies presented in this thesis should be made with caution, because all studies were observational and most (except **Chapters 3 and 5**) had a cross-sectional design, and reverse causality therefore cannot be ruled out. A cause could be defined as an antecedent event, condition or characteristic without which the disease event either would not have occurred at all or would have occurred at a later time (7). Causality is one of the most difficult concepts to study and cannot fully be tested in a cross-sectional design. Therefore, longitudinal studies are needed to further clarify the mechanisms underlying the associations reported in **Chapters 2,4,6 and 7**.

MEASUREMENTS OF THE MAIN DETERMINANTS

Body composition

Body composition was measured by means of dual energy x-ray absorptiometry (DXA) (**Chapters 2 and 6**). This enables a precise characterization of individuals' levels of fat as accumulated in different body regions (trunk vs. peripheral) and also of the levels of lean (muscle) mass and their independent, and potentially opposite, association with outcomes of interest (e.g. endothelial dysfunction, low-grade inflammation and hypercoagulability). We have examined associations between body composition and these outcomes by means of multivariate models, allowing quantification of the expectedly adverse effects of central or trunk fat but also of the potential beneficial effects of peripheral fat and lean masses (4, 8-10). Because the different fat measures are strongly associated with each other, it can be argued that adjustment of these measurements for each other in such multivariate models led to overcorrected models. However, although the regression coefficients changed considerably after adjustments of body composition variables for one other (e.g. Table 3 of **Chapter 6**), our models were not disturbed by multicollinearity (i.e. tolerance was >0.1. in all models).

In addition, trunk fat as assessed by DXA correlates highly with CT measures of visceral fat as with total abdominal fat in elderly (11) and younger adults (12), implying associations with subcutaneous fat in this region. However, DXA measures do not enable distinctions between subcutaneous and visceral fat in the trunk/abdominal region in elderly (11), and therefore do not allow direct inference about potential differential 'effects' of these two

fat depots on the outcome variables of our studies. Additional measures of such fat depots are still thus necessary for a more detailed understanding of the pathophysiological mechanisms linking different fat depots to metabolic and vascular health.

Definition of Metabolic Syndrome

The concept of MetS has, and still is, an ongoing subject of discussion, especially with respect to its pathogenesis and clinical usefulness. In **Chapter 4** we used MetS expressed as a continuous variable (i.e. mean of z-scores of each trait) to take into account the continuous nature between the MetS traits and CVD. This method is more robust and does not suffer from the problem of dichotomization of continuous risk variables (as disease risk does not start at a certain cut off point (13)), something for which the commonly used definitions of MetS such as those by the National Cholesterol Education Program (NCEP) or the International Diabetes Federation (IDF) have been criticized for (14, 15). Because all MetS traits show a graded association with cardiovascular risk, establishing specific cut-off values is an arbitrary decision, but useful from a clinical point of view (16).

In **Chapter 5**, the definition of MetS at changes in MetS status was based on the clustering of 3 or more traits defined based on its distribution within the AGAHLs study population (i.e. highest sex-specific quartiles of systolic or diastolic blood pressure, waist circumference, triglycerides, and glycated haemoglobin (HbA1c) and the lowest sex-specific quartile of high density lipoprotein (HDL) cholesterol), as measured at baseline. We used HbA1c, because fasting plasma glucose levels (FPG) were not available at baseline in the AGAHLs. Epidemiological evidence supports the use of HbA1c when FPG is missing, because HbA1c, as FPG, is strongly associated with CVD (17-20) and recently, HbA1c has also been recommended for the diagnosis of DM2 replacing the traditional oral glucose tolerance test (OGTT) values (21, 22). The use of cut-off values based on the distribution of traits within the study population (quartiles) potentially lead to some degree of misclassification and the estimated prevalence and incidence of the MetS may therefore not directly compare to those obtained with standard definitions applied to cohorts of comparable age. However, our estimates were comparable to those obtained in Cardiovascular Risk in Young Finns Study, a large population based cohort, where MetS was defined according to a standard definition (23).

Our approach allowed: 1) the identification of those individuals with the worst MetS profile (clustering of MetS traits that could not be explained by chance alone) at baseline and its change over time – by the use of 4 phenotypes: never, incident, recovery and

persistent; and 2) the comparison (and illustration) of arterial properties changes over time (between the ages of 36 and 42) by changes in MetS status over the same time period. With the use of (changes in) a continuous MetS score such arterial changes would not be clearly depicted, because in longitudinal analyses, individuals who have a stable high or a stable low MetS (continuous) score over time would have a similar change value (close to 0). Thus, despite the limited external validity, our approach served the purpose of identification of different phenotypes incorporating changes in MetS status over time, which was suitable for this study.

Definition of glucose metabolism status

Hoorn Study participants' glucose metabolism status, i.e. normal glucose metabolism (NGM), impaired glucose metabolism (IGM), including both those with impaired fasting glucose and/or impaired glucose tolerance) and DM2, was defined by means of a standard 75g OGTT according to the criteria defined by the World Health Organization (WHO) in 1999 (24), which was the relevant definition at the time (i.e. 2000-2001 follow-up definition). More recently, in 2009, an International Expert Committee (representing the American Diabetes Association (ADA), the European Association for the Study of Diabetes (EASD) and the IDF) has proposed the diagnosis of diabetes to be made primarily on the basis of HbA1c levels $\geq 6.5\%$ (47 mmol/mol) (21). Though the investigation of HbA1c levels in its continuous range is emphasized in recognition of its association with a continuum of risk for micro- and macro-vascular disease risk, values in the range of 6.0-6.5% (42-47 mmol/mol) are proposed as indicative of a 'high-risk state'. The use of these criteria for the definition of diabetes were not considered appropriate in our studies since indication for treatment during the 2000-2001 assessment of the Hoorn Study (i.e. the data used in our studies) were based on the criteria in use by then (WHO 1999).

However, when we compared the associations between glucose metabolism status (by using either the OGTT-based (as used in **Chapter 7**) or HbA1c-based definitions) and thrombin generation, the two definitions yielded similar results (**Table 1**). This implies that the HbA1c-based definition might also be applicable to our data. It should be noted however that, with the currently used cut off points, HbA1c is less sensitive (but more specific) in identifying individuals at high-risk for DM2 compared with the OGTT. Therefore, some individuals may have been misclassified as 'normal' with the HbA1c-based definition which potentially could have led to slightly underestimated associations.

Table 1. Associations between glucose metabolism status according to the old and new definitions and thrombin generation.

	<i>WHO 1999*</i> <i>-75g OGTT -</i>				<i>International Expert Committee</i> <i>(ADA, EASD, IDF 2009)</i> <i>- HbA1c -</i>			
	NGM (n=275)	IGM (n=176)	DM2 (n=293)	<i>p-value</i> <i>(IGM+DM2</i> <i>vs. NGM)</i>	Normal < 6.0% (n=305)	High-risk 6.0-6.5% (n=123)	DM2** ≥ 6.5% (n=316)	<i>p-value</i> <i>(IGM+DM2</i> <i>vs. NGM)</i>
Lag time (min)	3.98 ± 0.83	4.10 ± 0.80	4.14 ± 0.77	0.021	3.98 ± 0.81	4.09 ± 0.76	4.16 ± 0.79	0.008
Peak Height (nM)	182 ± 57	191 ± 58	189 ± 59	0.067	183 ± 56	189 ± 60	189 ± 58	0.137
ETP (nM*min)	1166 ± 222	1218 ± 237	1192 ± 238	0.034	1175 ± 214	1210 ± 255	1194 ± 240	0.171

Results are presented as means ± standard deviation

*adapted from Table 2 of **Chapter 7**; **group consists of all individuals with a HbA1c ≥6.5% or those who used antidiabetic drugs

WHO: World Health Organization; OGTT: oral-glucose tolerance test; NGM: normal glucose metabolism; IGM: impaired glucose metabolism; DM2: type 2 diabetes mellitus; ETP: endogenous thrombin potential

MEASUREMENTS OF THE OUTCOME VARIABLES

Endothelial function

The term endothelial dysfunction has been most often used to refer to impairment of the endothelium-dependent vasodilation capacity, but may be better characterized by changes of the endothelium, either in the basal state or after stimulation, which are inappropriate with regard to the preservation of organ function (25). The endothelium has many functions and actively regulates vascular tone and permeability, the balance between coagulation and fibrinolysis, the composition of the subendothelial matrix, the adhesion and extravasation of leukocytes, and inflammatory activity in the vessel wall (26). Therefore, there is no ideal single test to measure (non-invasively) endothelial function (27-29). Brachial artery flow-mediated vasodilation (FMD), as assessed by non-invasive ultrasound, is widely used to investigate the endothelial vasodilatory function. Measures of plasma levels of endothelial-derived regulatory proteins (biomarkers) are also commonly used. We have therefore used both measures to characterize as comprehensively as possible endothelial (dys)function among the participants of the Hoorn Study (**Chapter 2**). Each measure warrants some critical methodological appreciations, however.

Flow-Mediated Dilation (FMD)

Most often FMD is expressed as the change in diameter relative to the baseline diameter (D), i.e. D_{peak} after cuff-release – baseline D/baseline D, in %. A percentage change statistic is not appropriate because baseline diameter varies considerably between individuals and is inversely associated with FMD% (30, 31) i.e. the initial baseline D has the potential to introduce mathematical bias into the quantification of FMD (in %), with smaller vessels

appearing more reactive and vice versa (32). To circumvent this problem analyses of the *absolute* change in diameter (ΔD , in mm) adjusted for the baseline D, as a covariate, have been proposed (33, 34). Such analytical procedure applies well in the context of randomized controlled trials when any imbalances between individuals in baseline measures may be assumed to be random and unrelated to the ‘determinant’ of interest (35). However, it must be noticed that these assumptions do not hold in the context of observational research, where the determinant of interest (e.g. lean mass in Chapter 2) may itself be related with the baseline D, in which case adjustment for the baseline D may introduce some bias by inflating the true associations (36). For these reasons, and to enable a full appreciation, we have shown our results on the associations between body composition with FMD as expressed as ΔD (in mm) with and without adjustments for the baseline D (**Chapter 2**).

In addition, when an arbitrary time point or time window (most often after 60s of cuff release as in the original work by Celermajer et al (37)) is used to determine the FMD response, there can be significant underestimation of the true FMD (38). To circumvent this problem to some extent, our FMD protocol included 4 measurements of brachial D up to 5 min after cuff release (i.e. at 45s, 90s, 180s and 300s). D_{peak} was therefore determined as the maximal D after cuff release occurring at any of these time points (and for the vast majority of the subjects this occurred at 45 or 90s). These 4 measures also allowed us to estimate an incremental area under the FMD curve response (FMD_{AUC}) by means of integrals computation (**Figure 2**) (39), although the 4 time points may have not been enough to fully capture its contours and the true D_{peak} . Indeed, current automated diameter analysis systems enable the continuous measurement of post cuff release diameters at 3-5s time points enabling a more precise calculation of the FMD_{AUC} and the D_{peak} (40). These systems have revealed that the further apart the time points are the lower the FMD is, and therefore with our protocol we may have underestimated FMD. Likely this was non-differential and if anything led to an underestimation of the associations reported.

It is thought that the peak response mainly represents the nitric oxide (NO) and prostaglandin availability and that the overall area under the curve potentially represents other vasodilating factors such as endogenous hydrogen sulfide (H_2S), that functions as a neuromodulator and potentially has an effect on vascular diameter by relaxing vascular smooth muscle cells (41), and carbon monoxide (CO), which exerts a dilator influence on the cerebral circulation and is involved in active hyperaemia, autoregulation, hypoxic dilation, and counteracting vasoconstriction (42, 43). Although our findings shown in **Chapter 2** suggest that the beneficial impact of lean mass in elderly individuals on FMD

was mainly NO mediated (as no associations with FMD_{AUC} were observed – data not shown), the use of continuous measurements of D as of blood flow velocity (the stimulus) by modern systems may shed a better light into these issues and the whether FMD_{AUC} offers any additional clinical relevant information for the assessment of endothelial dysfunction (44).

Figure 2. Illustration of an FMD curve response as estimated by General Estimated Equations (GEE): by regressing the observed post-occlusion diameters data on baseline diameter and time (treated as dummy variable). With the use on all four post-occlusion diameter data (imputed when necessary) we also estimated an incremental area under the FMD curve (FMD_{AUC} , in mm*min) by means of integral computation (39).

Circulating biomarkers

In addition to the assessment of FMD we have measured several circulating biomarkers of endothelial dysfunction, i.e. soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular cell adhesion molecule-1 (sVCAM-1), soluble endothelial selectin (sE-selectin) and von Willebrand factor (vWf) and also of low-grade inflammation, i.e. C-reactive protein (CRP - twice), serum amyloid A (SAA), interleukin-6 and interleukin-8. Although the reproducibility of these measurements was good (45), these assessments were obtained recently by means of electro-chemiluminescence technology in frozen samples that have

been stored for approximately 10 years, with the exception of vWf and CRP, which were measured shortly after the Hoorn 2000-2001 examination (by ELISA methods). We can therefore not completely rule out that the long period of storage might have affected the absolute levels of these biomarkers. However, this could to a certain extent be excluded, because we also obtained CRP measurements with the most recent technology (in frozen samples) and, if anything we found higher rather than lower levels of CRP in these than in the prior assessments (ELISA) but a very high correlation between the two ($r > 0.9$), indicating that the rank between individuals was preserved and no differential error was introduced by storage time. The differences in concentrations are thus more likely due to the different methodologies and differences on the standards provided by the respective commercial kits, which have not yet been sufficiently standardized (46). Furthermore, we have previously shown that concentrations of sICAM-1, sVCAM-1, CRP and sE-selectin remained stable over a relatively short period (47) and others have confirmed stable concentrations of CRP after 3-years of storage (48).

Cardiovascular autonomic dysfunction (C-AD) and (micro)albuminuria

In **Chapter 3**, cardiovascular autonomic function was assessed with both the standard Ewing tests, which are widely used by diabetologists and newer methods based on heart rate variability and baroreflex sensitivity, which are more often used by cardiologists. We calculated an overall C-AD total score based on the results of these tests to characterize C-AD as comprehensively as possibly by a robust measure comprising its several dimensions. Given the moderate reproducibility of each test (49), analyzing the association between each test separately and cardiovascular mortality would more likely be affected by misclassification (probably random) and underestimate the risk than the C-AD total score. By combining the tests into an overall score we also avoided the potential problem of multiple testing (i.e. type I errors) that would have occurred when each test would have been analyzed separately.

The presence of (micro)albuminuria was defined on the basis of albumin-to-creatinine ratio measured in only one urine (first voided) sample for most of the Hoorn study participants. (Micro)albuminuria is quite variable from day to day and we cannot fully exclude the possibility that some subjects were misclassified. Ideally, measurement of albumin excretion in a 24-hour urine collection would provide more precise measures of individuals' albuminuria status. However, these collections are unpractical and inconvenient for patients. Furthermore, it has been shown that an albumin-to-creatinine ratio is at least as reliable as measuring the gold standard, 24-hour urinary albumin excretion, for prediction of cardiovascular morbidity and mortality (50).

Remodeling of the carotid artery

We used non-invasive ultrasound imaging to assess (changes in) properties of the carotid artery, i.e. lumen diameter (LD), inter-adventitial diameter (IAD) and intima-media thickness (IMT) to ascertain different types of remodeling processes related to the prevalence of the MetS in the Hoorn Study (**Chapter 4**) or with changes in MetS status in the AGAHLs (**Chapter 5**). These structural measurements were complemented with estimates of the haemodynamic forces exerted at the arterial wall (i.e. circumferential wall tension and stress), providing a more comprehensive view of the remodeling process than most of the studies thus far. The protocol and equipment used in assessment of these measurements were similar in both cohorts and within the baseline and follow-up assessments of the AGAHLs, and conform the international guidelines for user procedures (51, 52) with measures being obtained with very good levels of intra-observer (Hoorn Study, 1 observer) (53) and inter-observer (AGAHLs, 2 observers) reproducibility (3). In the AGAHLs, the arterial measurements were obtained approximately 6 years apart and by 2 different observers. Therefore, considerable efforts were made to ensure longitudinal reproducibility (i.e. pilot reproducibility study between observers involved in the baseline and the follow-up assessments, and use of image prints of exact measurement locations as obtained at baseline as aids in the follow-up measurements). Measurement errors cannot be fully ruled out, but most likely these led to an underestimation of the associations reported as observers were blinded to the individuals' MetS status. However, individuals' (central) obesity status, a key component of the MetS was of course unavoidably open to the observers' perception, and in a way this could have introduced some sort of differential bias.

Finally, a key limitation of ultrasonography is that it does not distinguish between the intima from the media layers of the carotid wall. Therefore, whether MetS-related increases in IMT reflect atherosclerosis (intima) or arteriosclerosis (media) cannot be made with certainty. In addition, and in view of more recent developments in arterial ultrasonography technology, all arterial measurements obtained in the Hoorn and AGAHLs cohorts were confined to a specific site (marked by a single M-line centered approximately 1 cm proximal to the carotid bifurcation) but did not cover its length. Recent echotracking systems allow measurements over the arterial length (54) and may thus be more precise.

(Hyper)coagulability - thrombin generation

The measurements of the thrombin generation parameters were performed in 2008 in plasma samples that were stored at -80° for 7 to 8 years, but that had not been thawed before the thrombin generation analyses were performed. It is not known to what extent storage time could have affected the levels of thrombin generation parameters as

reported in our studies. Although the Laboratory for Clinical Thrombosis and Haemostasis of the Maastricht University Medical Centre, where the measurements of thrombin generation parameters were performed, has been able to rule out the effects of approximately one year of storage time on *ex-vivo* thrombin generation, long-term effects, if any, remain unknown. If long-term storage introduced an error, most likely it was a systematic one since storage time was approximately the same for all plasma aliquots. In this case, we can rule out its influence on the associations (**Chapter 6**) or differences found between the different groups of glucose metabolism status (**Chapter 7**). If random errors were introduced, or even if storage time reduced the variation of the parameters, then the magnitude of the associations or differences between groups were most likely underestimated. Nevertheless, a better insight on the effects of (long) storage time need to be provided by newer biochemical assessment methods. In addition, standardization and definition of normal or high-risk ranges for the parameters obtained by such new methods are necessary to enable a clinical meaningful interpretation and comparability of data across different clinical populations.

In this line, we compared the thrombin generation parameters of individuals with NGM, IGM and DM2 as measured in the Hoorn study population (reported in **Chapter 7**) with the results of 134 healthy individuals who were also tested in the same lab (55) – **Table 2**. Clear differences in the absolute values of lag time, peak height and endogenous thrombin potential were observed between the healthy group and all of the groups in the Hoorn Study which could be attributable to age and risk factor differences between the healthy individuals and the individuals of the Hoorn Study.

The thrombin generation parameters measured in our population were measured without corn trypsin inhibitor (CTI). Corn trypsin inhibitor has been advocated to prevent contact activation, but is expensive and only acts when already present in the test tube when the blood is drawn. It was previously shown that CTI was not required at tissue factor concentrations >0.5 pM (note that in **Chapters 6** and **7** a concentration of 1 pM was used), though this was only tested in normal pooled plasma, not in patients (56).

Finally, the thrombin generation parameters were measured in platelet-poor plasma, which, in the context of arterial thrombosis, is probably not the most accurate reflection of the ‘true’ coagulability state, as platelets play an important role in the aetiology of atherothrombosis. At the moment, new thrombin generation assays are under development in whole blood, which may provide more accurate assessments of thrombin generation in the context of atherothrombosis. Therefore, the associations examined in

Chapters 6 and 7 should be reassessed in platelet-rich plasma before a statement can be made about the clinical usefulness of the thrombin generation assay.

Table 2. Comparison of thrombin generation parameters of healthy individuals and Hoorn population (stratified by glucose metabolism status)

	Healthy group (n=134)*	Hoorn Study		
		NGM (n=275)	IGM (n=176)	DM2 (n=293)
Age, years (range or SD)	54 [20 to 90]	68.5 ± 6.0	70.3 ± 6.5	67.6 ± 8.0
Sex, % female	52.1	52.7	49.4	49.1
Lag time (min)	5.84 ± 1.65	3.98 ± 0.83	4.10 ± 0.80	4.14 ± 0.77
Peak Height (nM)	116 ± 50.8	182 ± 57	191 ± 58	189 ± 59
ETP (nM*min)	995 ± 289	1166 ± 222	1218 ± 237	1192 ± 238

* J Thromb Haemost 2008; 6: 125–31. Data are means ± SD or frequencies, except for age in the healthy group [mean and range].

PATHWAYS - CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

To better understand the pathogenesis of CVD and improve risk estimation, considerable research effort has been put on the identification of new factors or biomarkers that, in addition to the traditional risk factors, are associated with CVD. The studies comprised in this dissertation intended to contribute to that effort. We investigated several intermediate phenotypes of CVD (such as endothelial dysfunction, C-AD, (maladaptive) remodeling of the carotid remodeling and (hyper)coagulability). Our findings elucidated some potential pathways through which individuals with metabolic disorders such as central obesity, MetS and DM2 may be at increased risk for CVD (**Figure 2**).

Although primary prevention of obesity and related metabolic disease remains the best strategy to prevent CVD at the population level, treatment interventions targeting the pathways we have elucidated may constitute a way to improve the health status of individuals who are obese, have MetS and/or DM2 and have difficulties to recover from these states. To recover from these conditions considerable lifestyle changes requirements are often needed (e.g. severe calorie restriction, dietary pattern changes or more exercise) when, at the same time, such individuals may be less amenable to such interventions (e.g. due to more physical limitations, respiratory problems, food habits etc.), especially at older ages.

Figure 2. Pathways of risk investigated/identified in the present thesis (thick arrows). Note: model is simplified and does not include all (and there are many other) possible pathways based on available evidence from other studies.

The observation that, in young individuals who recovered from MetS, all carotid artery properties were restored to levels that no longer differed from those who never developed MetS is supportive of the value of primary prevention. These findings also suggest that reversing MetS status could, at least in part, prevent maladaptive arterial remodeling and thereby decrease stroke risk. Still, this hypothesis is dependent on prospective studies, preferably randomized controlled trials, investigating the extent to which ‘curing’ MetS reduces the incidence of stroke in these individuals and how much of this beneficial effect can be attributed to favourable changes in arterial (remodeling) properties.

The observation, in elderly individuals, that the widening of the arterial diameter was mainly dependent on blood pressure (**Chapter 4**), suggests that the pulsatile component of blood pressure exerts a fatiguing effect on the load-bearing elements of the arterial wall (i.e. elastin and collagen) (57). In agreement with this, we observed that not only arterial widening but also the intrinsic elastic properties of the carotid artery wall (as estimated by Young’s elastic modulus), were already impaired at baseline among young

adults with MetS and preceded the subsequent steeper increases in IMT among those who remained with MetS at follow-up (**Chapter 5**). These findings support the view that arterial enlargement and stiffening may precede and stimulate subsequent wall thickening to restore the levels of wall tensile stress. It has been suggested that arterial remodeling could primarily reflect an adaptive mechanism of the artery to restore local hemodynamic conditions to an equilibrium (58, 59). Our longitudinal study among young individuals without overt signs of atherosclerosis served thus as an appropriate model to confirm this hypothesis. Still, the process of arterial remodeling related to (persistent) MetS remained maladaptive and other MetS components than blood pressure, such as central obesity and disturbed glucose metabolism, seemed to contribute to this as well.

Our results also suggest that in elderly individuals not only (central) obesity but also loss of peripheral lean mass (at any given level of body fatness) may affect endothelial dysfunction, although these associations may be complementary in the sense that central fatness was adversely associated with circulating biomarkers of endothelial dysfunction, whereas lean mass was adversely associated with FMD (**Chapter 2**). Therefore, our findings emphasize that sarcopenia could be an additional driving factor for the decline in endothelial function in elderly. This is a novel observation enabled by our comprehensive assessment and investigation of body composition as a determinant of endothelial dysfunction. Indeed, most studies thus far focused on the adverse role of (central) fatness on endothelial dysfunction, not providing any information on the concomitant role of muscle mass. The pathobiological mechanisms linking muscle mass to endothelial dysfunction are also not clear and may be distinct from those linking (central) fatness to endothelial dysfunction. We hypothesized that low-grade inflammation and/or insulin resistance could be involved, and although these explained the associations between (central) fatness and endothelial dysfunction (as expressed by the circulating biomarkers), they did not explain the lean mass-FMD associations. Factors specifically related to sarcopenia, such as androgen deficiency or oxidative stress, may thus be involved (60). However, the link between lean mass and endothelial dysfunction may also be non-causal, but just a reflection of common underlying factors, such as physical activity and/or nutrition status. Therefore, further studies are needed to clarify this pathway. Implications of these observations are that lifestyle (diet and/or exercise) or medicinal interventions targeting the elderly should not only be focused, as they often are, on loss of excessive weight (which is frequently accompanied by loss of muscle mass), but also on the preservation of muscle mass. In addition, the evaluation of the beneficial effects of diet and/or exercise lifestyle interventions on endothelial dysfunction should also inform on the extent to which these can be attributed to changes in muscle mass rather than, or in addition to, body fatness.

We found that the increase in thrombin generation observed in individuals with IGM and/or DM2 could be, to a great extent, explained by their elevated adiposity-related low-grade inflammation (**Chapter 7**). In this line, abdominal obesity and low-grade inflammation were identified as the main determinants of enhanced thrombin generation in young adults (61). There is abundant evidence for a close interaction between inflammation and coagulation systems and a bidirectional association between the two has also been proposed (62, 63). Which direction is predominant, if any, is not clear. Thrombin is associated with a stimulation of pro-inflammatory cytokines (64-67). We hypothesized that adiposity-related increases in low-grade inflammation could lead to higher thrombin generation, a hypothesis that was to a considerable extent (up to 55%) confirmed by our data, although confined to women (Table 4 of **Chapter 6**). We have also investigated the alternative hypothesis, i.e. that body fatness leads to thrombin generation, which in turn leads to inflammation. These analyses showed that, in women, the associations between body fatness and hsCRP were only very slightly attenuated (by ~12%) when further adjusted for (parameters) of thrombin generation. Nevertheless, due to the cross-sectional and observational nature of the study we cannot fully discard the possibility that that low-grade inflammation and thrombin generation are both elevated as a consequence of (central) obesity without necessarily being causally related to each other. Further mechanistic studies investigating the cross-talk, if any, between hsCRP, but also other pro-inflammatory adipose-tissue-related factors, such as interleukin-6, sICAM1, serum amyloid A and leptin, and thrombin generation are needed.

Recently, some studies have reported increased levels of thrombin formation in individuals with echogenic carotid plaques (68), or with increased IMT (69). In addition, thrombin generation was associated with incident ischaemic stroke although not with myocardial infarction (70), and notably, this association was present in women but not in men. Further studies are thus necessary to establish whether this test is a useful predictor of individuals' risk of atherothrombotic disease in particular, and any potential effect modification by sex herein. Prospective data from the Hoorn study may be useful in this regard.

The association between microalbuminuria and cardiovascular mortality was not attenuated by cardiovascular autonomic dysfunction (C-AD) and also the association between C-AD and cardiovascular mortality was not attenuated by microalbuminuria, though C-AD and microalbuminuria were modestly associated with each other (**Chapter 3**). This implies that other biological mechanisms underlie these associations. Several mechanisms linking C-AD to cardiovascular mortality have been proposed (e.g., QT interval prolongation leading to arrhythmias and silent myocardial ischaemia) (71). In this line C-

AD has been linked to incident CVD, especially in individuals at high risk (DM2, hypertension, or prior CVD) (72, 73). It may be useful to test for and treat C-AD (e.g., with exercise training, beta blockers or lipid-lowering drugs) in populations at high risk of cardiovascular mortality. Finally, in individuals with DM2 and C-AD, tight glycaemic control might prevent deterioration of heart rate variability (74). On the other hand, it is unlikely that microalbuminuria itself *causes* cardiovascular mortality; instead, it may be linked to CVD by a common pathophysiological process, such as endothelial dysfunction, or low-grade inflammation or by residual confounding by yet to be discovered factors (75). For instance, a cross-sectional study suggested that increased thrombin generation in individuals with DM2 explains part of the association between albuminuria and CVD in these individuals (76). This needs to be investigated in future prospective studies.

Finally, whether monitoring of the levels of the surrogate markers discussed above (e.g. levels of biomarkers of endothelial dysfunction or thrombin generation, arterial remodeling properties), may be useful for the clinical management of elderly individuals with (central) obesity, or related metabolic diseases, still depends on the extent to which improvement of the levels of these markers result in an improved prognosis (77).

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The background of the entire page is a dense, close-up pattern of heart-shaped stones. The stones are in two colors: white and a light grey. They are scattered across the entire surface, creating a textured, romantic, and minimalist aesthetic. The lighting is soft, highlighting the smooth, polished surfaces of the stones.

Samenvatting

Samenvatting voor niet-ingewijden

Diabetes mellitus type 2 (DM2), oftewel 'ouderdomssuikerziekte' is een steeds vaker voorkomende aandoening. Dit heeft onder andere te maken met de vergrijzing en onze Westerse leefstijl, waardoor steeds meer mensen te dik worden. Overgewicht kan ervoor zorgen dat het lichaam minder gevoelig wordt voor insuline (ook wel insulineresistentie genoemd) wat weer kan leiden tot DM2. Deze verminderde insulinegevoeligheid treedt in het bijzonder op wanneer het teveel aan vetweefsel zich centreert op de buik, het zogenaamde appelfiguur.

De belangrijkste oorzaak van ziekte en sterfte in patiënten met prediabetes en DM2 is hart- en vaatziekten. Dit komt ondermeer doordat er vaak gelijktijdig sprake is van een aantal risicofactoren op hart- en vaatziekten zoals een toegenomen (buik)vetmassa, verminderde insulinegevoeligheid, een te hoge bloeddruk, een te laag 'goed' cholesterol (bij normaal 'slecht' cholesterol) en teveel vetten (triglyceriden) in de bloedbaan. Indien men een toegenomen (buik)vetmassa heeft en tevens 2 of meer andere van deze factoren gelijktijdig bezit, wordt dit overigens het metabool syndroom (MetS) genoemd. Mensen met het MetS hebben een 5-voudig verhoogd risico op het ontwikkelen van DM2 en een 2-voudig verhoogd risico op het krijgen van hart- en vaatziekten. Echter, bovengenoemde risicofactoren kunnen het verhoogde risico op hart- en vaatziekten in patiënten met MetS en DM2 niet volledig verklaren. Onderzoek naar andere potentiële mechanismen die het verhoogde risico op hart- en vaatziekten in deze patiënten (gedeeltelijk) kunnen verklaren is derhalve van belang zodat preventie programma's en behandelstrategieën hierop kunnen worden aangepast.

Het belangrijkste doel van dit proefschrift was om de huidige kennis met betrekking tot de onderliggende mechanismen op hart- en vaatziekten in patiënten met toegenomen (buik)vetmassa, MetS en DM2 te onderzoeken. In **Hoofdstuk 1** van dit proefschrift worden de klinische setting en de probleemstellingen geïntroduceerd. Hierbij hebben we gebruik gemaakt van 2 grote studies: de Hoorn Studie, een Nederlandse studie naar stoornissen in de glucosehuishouding en de complicaties hiervan in mensen tussen de 50 en 75 jaar bij aanvang van de studie, en de Amsterdam Growth and Health Longitudinal Study (AGAHLS) een studie naar leefstijl risicofactoren in opgroeiende jongeren die gedurende lange tijd vervolgd worden.

In **Hoofdstuk 2** wordt het verband tussen lichaamsvetverdeling en endotheeldisfunctie in ouderen onderzocht. Endotheelcellen zitten aan de binnenkant van de vaatwand van de slagader en geven veranderingen in de circulatie door aan de gladde spiercellen van het

bloedvat. Op deze manier kan het fysiologische evenwicht worden gehandhaafd. Veranderingen in de functie van deze endotheelcellen met dientengevolge orgaandisfunctie, wordt ook wel endotheeldisfunctie genoemd. Het is in eerdere onderzoeken aangetoond dat endotheeldisfunctie een goede voorspeller is voor toekomstige hart- en vaatziekten. Ook weten we dat naarmate mensen ouder worden endotheeldisfunctie toeneemt. Mogelijk spelen veranderingen in de lichaamsbouw hierbij een rol. In **Hoofdstuk 2** hebben we endotheeldisfunctie op 2 verschillende manieren gemeten: ten eerste dynamisch door het meten van de mate van verwijding van het bloedvat na een periode van bloedleegte (bloedstroomgemediteerde dilatatie) en ten tweede door het meten van vaatwandstoffen in het bloed (biomarkers). Vervolgens hebben we onderzocht waardoor de gevonden verbanden tussen lichaamsvetverdeling en endotheeldisfunctie verklaard konden worden. Als mogelijke verklaring hebben we allereerst gekeken naar de rol van laaggradige ontsteking. Dit is een vorm van ontsteking die enkel meetbaar is in het bloed maar niet tot ontstekingsverschijnselen zoals koorts e.d. leidt. Hiervan is bekend dat dit het risico op hart- en vaatziekten verhoogt. Tevens hebben we de rol van verminderde insulinegevoeligheid onderzocht. De belangrijkste bevindingen waren dat hoe meer (buik)vet men had des te slechter de endotheelfunctie was. Dit werd grotendeels verklaard door de hierboven genoemde laaggradige ontsteking en verminderde insulinegevoeligheid. Verder was er een beschermend verband tussen vetvrije (dus spier-)massa van de armen/benen en endotheeldisfunctie, wat dus mogelijk impliceert dat het behouden van spiermassa in ouderen tevens van belang is voor het behoud van endotheelfunctie.

In **Hoofdstuk 3** hebben we vervolgens gekeken naar het verband tussen kleine hoeveelheden eiwit in de urine (microalbuminurie) en sterfte aan hart- en vaatziekten. Het aanwezig zijn van kleine hoeveelheden eiwit in de urine is sterk geassocieerd met het later optreden van hart- en vaatziekten. Hoe dit komt is echter onbekend. Een mogelijke verklaring hiervoor zou het verminderd functioneren van het onwillekeurige zenuwstelsel (cardiovasculaire autonome disfunctie (C-AD)) kunnen zijn. Het is namelijk bekend dat er een verband bestaat tussen zowel C-AD en microalbuminurie als tussen C-AD en hart- en vaatziekten. Bovengenoemde hypothese hebben we in **Hoofdstuk 3** onderzocht waarbij bleek dat zowel microalbuminurie als C-AD onafhankelijk van elkaar gerelateerd waren aan sterfte aan hart- en vaatziekten, wat suggereert dat beiden via verschillende biologische mechanismen leiden tot hart- en vaatziekten.

Herseninfarcten komen vaker voor bij mensen met het MetS. De onderliggende oorzaak hiervoor is onduidelijk maar mogelijk spelen veranderingen van de halsslagader hierin een rol. Deze veranderingen zijn erop gericht om de slagader te beschermen tegen een te

hoge spanning op de vaatwand. In **Hoofdstuk 4** hebben we gekeken of er in mensen met het MetS (maar zonder DM2) sprake is van structurele en functionele veranderingen van de halsslagader. Er bleek inderdaad een verband te zijn tussen het MetS en veranderingen van de vaatwand van de halsslagader. Dit verband kon echter niet verklaard worden door laaggradige ontsteking of verminderde insulinegevoeligheid en slechts gedeeltelijk door de hogere bloeddruk die deze mensen gemiddeld genomen hebben.

Vervolgens is in **Hoofdstuk 5** onderzocht of veranderingen in MetS status in jong volwassenen geassocieerd zijn met veranderingen in vaateigenschappen van de halsslagader. De belangrijkste bevindingen van deze studie waren dat “genezing” van het MetS in jong volwassenen gerelateerd was aan verbetering van zowel de structurele als functionele veranderingen van de halsslagader. Dit suggereert dus dat het genezen van MetS mogelijk het risico op herseninfarcten kan verminderen door gunstige effecten op de halsslagader. Deze hypothese zal in toekomstige studies verder uitgezocht moeten worden.

Teveel aan (buik)vetmassa is gerelateerd aan zowel trombose in het slagaderlijke systeem (hart- en vaatziekten) als trombose in de aders (bijv. trombosebeen). Een mogelijke verklaring hiervoor kan zijn een toegenomen stolbaarheid van het bloed in mensen met een toegenomen (buik)vetmassa. In **Hoofdstuk 6** wordt derhalve het verband tussen (buik)vetmassa en toegenomen stolbaarheid van het bloed (gemeten d.m.v. een trombinegeneratietest) onderzocht. In deze studie bleek dat er in vrouwen wel een verband is tussen (buik)vetmassa en trombinegeneratie, maar in mannen niet. Het is bekend dat vrouwen na de overgang meer laaggradige ontsteking hebben dan mannen. De gevonden verbanden tussen (buik)vetmassa en trombinegeneratie in vrouwen werden ook voor een groot gedeelte (ongeveer 55%) hierdoor verklaard. Een andere verklaring kan gezocht worden in hormonale verschillen tussen mannen en vrouwen. Dit valt echter buiten het bestek van dit proefschrift en zal in toekomstige studies onderzocht moeten worden. Vervolgens wordt in **Hoofdstuk 7** gekeken of bij patiënten met prediabetes of DM2 een verband gevonden werd met trombinegeneratie. Tevens werd bekeken of een dergelijk verband verklaard kon worden door bekende risicofactoren op hart- en vaatziekten. In deze studie vonden we dat de relatie tussen mensen met prediabetes of DM2 en trombinegeneratie slechts 4% hoger was dan die van mensen met een normale glucosehuishouding. Bovendien was deze relatie voor een groot gedeelte toe te schrijven aan de toegenomen (buik)vetmassa en daarmee geassocieerde laaggradige ontsteking in deze groep patiënten. Derhalve lijkt deze test niet de meest geschikte test om toegenomen stolbaarheid in deze patiëntengroepen te meten.

Tot slot, worden in **Hoofdstuk 8** de belangrijkste bevindingen van dit proefschrift samengevat en bediscussieerd. Hierbij worden methodologische kanttekeningen geplaatst en aanbevelingen gedaan voor de toekomst. Het voorkomen van vetzucht en gerelateerde ziekten (primaire preventie) is de beste manier om hart- en vaatziekten op populatieniveau aan te pakken. In dit proefschrift hebben we een aantal mogelijke mechanismes/routes onderzocht waarop behandelstrategieën zouden kunnen worden ingezet. Hierbij kun je denken aan leefstijl aanpassingen (afvallen, meer bewegen) of toch door middel van medicijnen. De toekomst zal ons moeten leren of het meten van de hierboven bediscussieerde surrogaatmarkers voor hart- en vaatziekten zinvol zijn voor de klinische behandeling van oudere patiënten met toegenomen (buik)vetmassa en gerelateerde stofwisselingsziekten zoals DM2. Eerst is het zaak dat wordt aangetoond dat verbetering van deze markers ook daadwerkelijk leidt tot minder hart- en vaatziekten.

Dankwoord

Dankwoord

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About the author

Hanneke Johanna Bernardina Huberta Beijers was born on March 29th 1982 in Nijmegen, the Netherlands. During her childhood she lived in Asten-Heusden, a small village in Noord-Brabant. After graduating from secondary school (Varendonck College Asten) in 2000, she started her study Medicine at the University of Maastricht. During her study she followed several internships abroad (Australia, Belgium, Surinam). After she graduated in 2006, she worked for one and a half year as a resident at the department of internal medicine at the Amphia Hospital Breda. In 2008 she started this AGIKO project, which was a collaboration project between the Catharina Hospital Eindhoven and Maastricht University Medical Centre. During this project she followed several statistical and epidemiological courses. Since 2010, she is also working as a resident at the department of internal medicine at the Catharina Hospital Eindhoven. From February 2014 she will be working as a resident Endocrinology at the Nijmegen University Medical Centre.

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