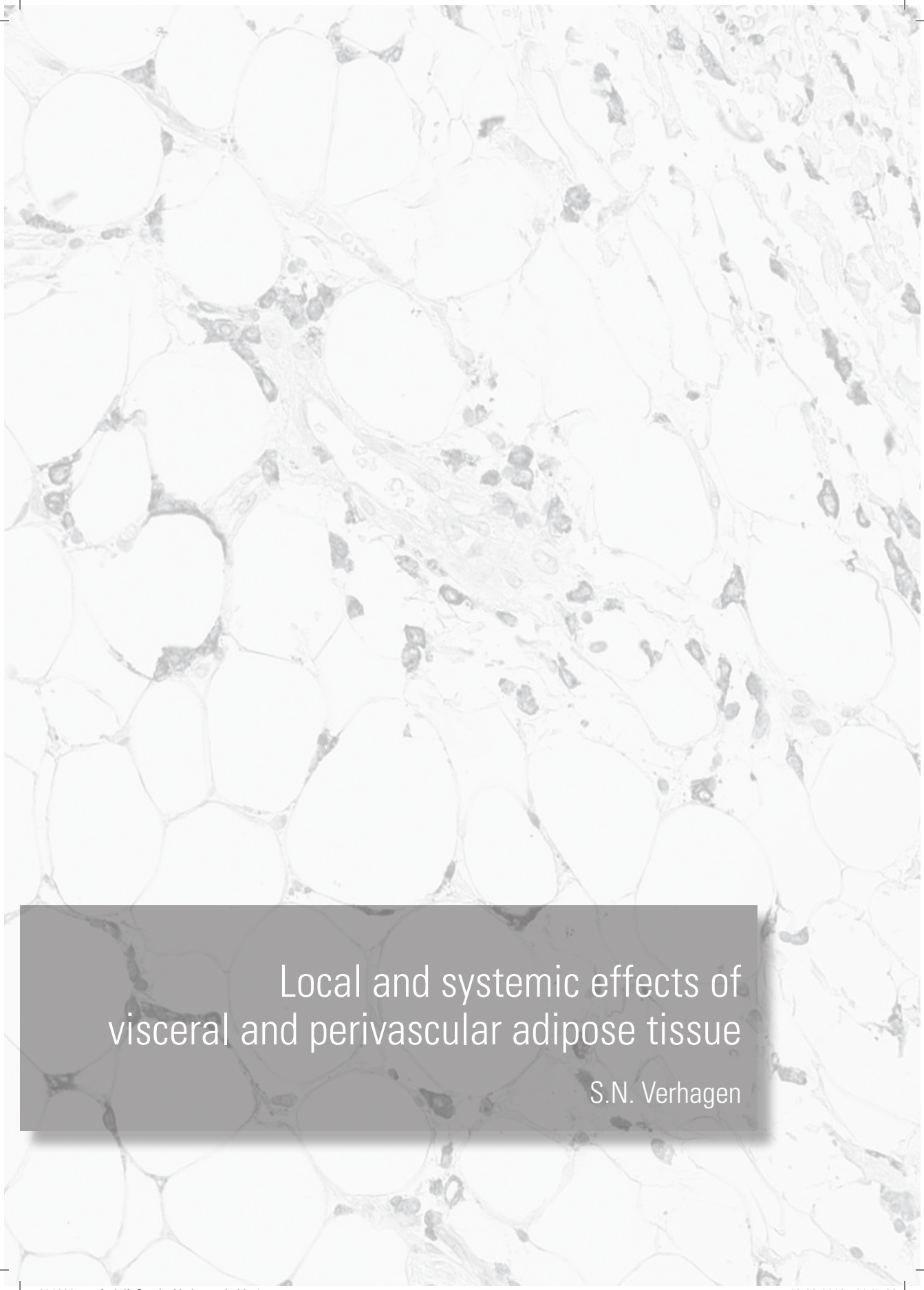

Local and systemic effects of visceral and perivascular adipose tissue

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Local and systemic effects of visceral and perivascular adipose tissue

Lokale en systemische gevolgen van visceraal en perivasculair vetweefsel
(met een samenvatting in het Nederlands)

Proefschrift

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CONTENTS

PART I

Chapter 1

General introduction

Obesity induces a pro-inflammatory state

Obesity, defined as a body mass index above 30 kg/m^2 is, apart from being associated with type 2 diabetes and cancer, now recognized as a risk factor for cardiovascular disease[1;2]. The rapid increase in the incidence rate of obesity begins to level off in the United States[3], although the current prevalence of obesity results in a considerable disease burden globally[4-6].

Adipose tissue consists of adipocytes and a stromal fraction containing pre-adipocytes, capillary endothelial cells and leukocytes, mainly macrophages. The different cell types present in adipose tissue are able to secrete pro-and anti-inflammatory cytokines[7]. In case of obesity, high levels of pro-inflammatory adipokines, some of them almost exclusively produced by adipocytes, can be measured in the systemic circulation[8-10]. Many of these adipokines are involved in inflammation and insulin resistance[11;12].

Adipose tissue depots

Adipose tissue is mainly located around the intra-abdominal organs (visceral adipose tissue) and subcutaneously (subcutaneous adipose tissue). The systemic pro-inflammatory activity is different between adipose tissue depots[13;14]. C-reactive protein, a well-known acute phase protein, is highly associated with the amount of visceral adipose tissue, but to a lesser extent with subcutaneous adipose tissue thickness[15]. The adipose tissue surrounding the heart, epicardial adipose tissue, secretes more MCP-1, tumor necrosis factor-α and interleukin-6 than abdominal visceral and subcutaneous adipose tissue[16;17]. Adipose tissue surrounding arteries, such as the aorta and the coronary arteries, is referred to as perivascular adipose tissue. Perivascular adipose tissue is most often part of other adipose tissue depots, such as epicardial adipose tissue or mesenterial adipose tissue. However, perivascular adipose tissue is different from adipose tissue that lies further away from the arterial wall even if it is located within the same adipose tissue depot[14;18]. Adipocytes in perivascular adipose tissue are more irregular in shape and smaller than epicardial adipocytes[18]. Furthermore secretion of pro-inflammatory cytokines is higher in perivascular than in epicardial adipose tissue[18].

Adipose tissue in relation to atherosclerosis

Atherosclerosis is a multifactorial disease. Classical risk factors for cardiovascular disease have been identified in cohort studies. These risk factors include smoking, high plasma cholesterol levels and hypertension, but also obesity has been identified as an independent risk factor[19-21]. Subsequently, therapies have been developed to control for these cardiovascular risk factors, thereby reducing atherosclerotic risk. Obesity, however, is difficult to manage, mainly because life style changes such as exercise training and a decrease in caloric intake are usually difficult to sustain[22]. The focus of obesity management is on reducing weight. However, it is not the quantity of adipose tissue but the function of adipose tissue, with metabolic consequences such as inflammation, insulin resistance, dyslipidemia and elevated blood pressure that are strongly related with the occurrence of cardiovascular diseases. Currently, inflammatory cells and cytokines involved in adipose tissue dysfunction are being scrutinized as new therapeutic targets[23;24].

Most major arteries that are typically affected by atherosclerosis such as the aorta and the coronary arteries, are surrounded by perivascular adipose tissue. Over the past few years, the presence of pro-inflammatory mediators in the tissues surrounding the vessel wall has led to a hypothesis of "outside to inside signaling" accelerating the atherosclerotic process inside arteries[17;25]. Secreted adipokines may diffuse into the arterial wall leading to attraction of macrophages, endothelial

dysfunction and smooth muscle cell proliferation. This can be described as a paracrine signal sent by the adipose tissue cells. To investigate the possibility of induction of atherosclerosis from outside of the vascular wall, cytokines have been applied on the outer vascular wall in animal studies[26;27]. Atherosclerotic plaque development in pigs was enhanced by extravascular treatment with IL-1β or MCP-1[26;27]. In addition, the cytokine expression profiles of patients with and without coronary artery disease have been compared[16;28;29]. Production of the pro-inflammatory adipocytokines IL-6, TNF-α, and leptin was increased in epicardial adipose tissue of patients with coronary artery disease than in patients without coronary artery disease[16;28], whereas adiponectin production was lower[16;29]. All this research is in favor of the hypothesis of paracrine signaling by perivascular adipose tissue potentially stimulating the process of atherosclerosis.

Adipose tissue in relation to insulin resistance

Obesity and the metabolic syndrome are highly associated with insulin resistance in the general population and also in patients with clinically manifest arterial disease[30;31]. An increase in (visceral) adipose tissue is accompanied by metabolic disturbances in the majority of subjects. The metabolic syndrome according to the ATPIII NECP criteria is defined as the presence of at least 3 of the following criteria: abdominal obesity, high blood pressure, low plasma HDL, elevated plasma triglycerides and an increased fasting glucose[32]. Although the clinical relevance of the metabolic syndrome has been under debate[33], it is a well validated way to describe the clustering of the metabolic disturbances associated with abdominal obesity[32;34]. Also, the metabolic syndrome is associated with increased cardiovascular risk in various groups of patients[35;36].

Because of the close relation between inflammation, insulin resistance and other metabolic disturbances associated with obesity, the independent association of these factors with atherosclerosis and insulin resistance is often a subject of debate. In this thesis we aim to unravel part of these interrelations in a population of patients with clinically manifest arterial disease[37].

Objectives of this thesis

The main aim of this thesis is twofold: to determine whether adipose tissue around arteries is involved in the local development of atherosclerosis (**Part 1**) and to identify the role of inflammation and the metabolic syndrome in the development of type 2 diabetes mellitus and atherosclerosis in patients with manifest arterial disease (**Part 2**)

The objectives of this thesis are:

Part 1

1. to determine whether the quantity of perivascular adipose tissue and histological characteristics of inflammation in perivascular adipose tissue are associated with local atherosclerosis (chapter 3 and 5)
2. to determine whether adipocytokine secretion of perivascular adipose tissue near stenotic coronary arteries is different from perivascular adipose tissue near non-stenotic coronary arteries (chapter 4)
3. to determine whether absence of perivascular adipose tissue in segments of coronary arteries with an intra-myocardial course is related to a reduction of coronary atherosclerosis (chapter 6)

Part 2

4. to determine and quantify the relationship between insulin resistance and recurrent arterial events and to evaluate the role of the individual components of the metabolic syndrome in this relationship (chapter 7)
5. to determine and quantify the relationship between inflammation and the occurrence of type 2 diabetes mellitus in patients with manifest arterial disease (chapter 8)

Outline of this thesis

In **part 1** of this thesis the relation between perivascular adipose tissue and atherosclerosis is addressed. In **chapter 2** the current literature on adipose tissue surrounding the heart is reviewed and definitions concerning the adipose tissue depots are set. Furthermore, characteristics of perivascular adipose tissue are described and literature on the relation with atherosclerosis is reviewed.

The relation between adipose tissue inflammation and atherosclerosis has been investigated thoroughly over the past decade. However, most of these studies describe differences between and not within subjects, because paracrine effects of perivascular adipose tissue are difficult to investigate in an experimental setting in humans. To evaluate whether perivascular adipose tissue may contribute to atherosclerosis of the adjacent vessel we performed several studies evaluating local characteristics of perivascular adipose tissue in relation to local atherosclerosis. In **chapter 3** we have examined the relation between the amount of perivascular adipose tissue and atherosclerotic plaque size in cross sections of the left anterior descending coronary artery (LAD) obtained post mortem. In addition we have explored the relationship between histological characteristics of inflammation in perivascular adipose tissue and atherosclerotic plaque composition. In **chapter 4** the inflammatory characteristics of perivascular adipose tissue in terms of adipocytokine secretion were further elaborated and related to atherosclerosis, by harvesting perivascular adipose tissue near stenotic coronary artery segments and near coronary artery segments without stenosis. In addition studying the number of macrophages in perivascular adipose tissue in **chapter 3**, macrophage polarization in perivascular adipose tissue in relation to atherosclerotic plaque composition was investigated in the same LAD cross sections (**chapter 5**). Perivascular adipose tissue is absent at coronary artery segments with an intra-myocardial course, often referred to as a myocardial bridge. In **chapter 6** the relation between myocardial bridges and absence of coronary atherosclerosis is evaluated in coronary artery segments covered by a myocardial bridge and matched coronary artery segments without a myocardial bridge.

Part 2 of this thesis focuses on adipose tissue in relation to systemic inflammation and insulin resistance. In **chapter 7** the relation between insulin resistance and the occurrence of a second vascular event in patients with manifest arterial disease was determined and the role of the metabolic syndrome and inflammation in this relation was further elucidated. In **chapter 8** the influence of visceral and subcutaneous adiposity on the CRP induced risk of type 2 diabetes was explored. In **chapter 9**, the main findings of the studies concerning perivascular adipose tissue and the studies concerning systemic inflammation and insulin resistance are discussed. Finally, a summary of the results presented in this thesis is given in **chapter 10**.

REFERENCE LIST

1 Haslam DW, James WP. Obesity. Lancet 2005; 366(9492):1197-1209.

2 Mokdad AH, Marks JS, Stroup DF, Gerberding JL. Actual causes of death in the United States, 2000. JAMA 2004; 291(10):1238-1245.

3 Flegal KM, Carroll MD, Kit BK, Ogden CL. Prevalence of obesity and trends in the distribution of body mass index among US adults, 1999-2010. JAMA 2012; 307(5):491-497.

4 Caballero B. The global epidemic of obesity: an overview. Epidemiol Rev 2007; 29:1-5.

5 Ford ES, Mokdad AH. Epidemiology of obesity in the Western Hemisphere. J Clin Endocrinol Metab 2008; 93(11 Suppl 1):S1-S8.

6 Prentice AM. The emerging epidemic of obesity in developing countries. Int J Epidemiol 2006; 35(1):93-99.

7 Spencer M, Unal R, Zhu B, Rasouli N, McGehee RE, Jr., Peterson CA et al. Adipose Tissue Extracellular Matrix and Vascular Abnormalities in Obesity and Insulin Resistance. J Clin Endocrinol Metab 2011.

8 DeClercq V, Taylor C, Zahradka P. Adipose tissue: the link between obesity and cardiovascular disease. Cardiovasc Hematol Disord Drug Targets 2008; 8(3):228-237.

9 Sattar N, Wannamethee G, Sarwar N, Tchernova J, Cherry L, Wallace AM et al. Adiponectin and coronary heart disease: a prospective study and meta-analysis. Circulation 2006; 114(7):623-629.

10 Shibata R, Ouchi N, Murohara T. Adiponectin and cardiovascular disease. Circ J 2009; 73(4):608-614.

11 Qasim A, Mehta NN, Tadesse MG, Wolfe ML, Rhodes T, Girman C et al. Adipokines, insulin resistance, and coronary artery calcification. J Am Coll Cardiol 2008; 52(3):231-236.

12 Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003; 112(12):1821-1830.

13 Fried SK, Bunkin DA, Greenberg AS. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. J Clin Endocrinol Metab 1998; 83(3):847-850.

14 Rittig K, Dolderer JH, Balletshofer B, Machann J, Schick F, Meile T et al. The secretion pattern of perivascular fat cells is different from that of subcutaneous and visceral fat cells. Diabetologia 2012.

15 Faber DR, van der Graaf Y, Westerink J, Visseren FL. Increased visceral adipose tissue mass is associated with increased C-reactive protein in patients with manifest vascular diseases. Atherosclerosis 2010; 212(1):274-280.

16 Cheng KH, Chu CS, Lee KT, Lin TH, Hsieh CC, Chiu CC et al. Adipocytokines and proinflammatory mediators from abdominal and epicardial adipose tissue in patients with coronary artery disease. Int J Obes (Lond) 2008; 32(2):268-274.

17 Mazurek T, Zhang L, Zalewski A, Mannion JD, Diehl JT, Arafat H et al. Human epicardial adipose tissue is a source of inflammatory mediators. Circulation 2003; 108(20):2460-2466.

18 Chatterjee TK, Stoll LL, Denning GM, Harrelson A, Blomkalns AL, Idelman G et al. Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. Circ Res 2009; 104(4):541-549.

19 Hubert HB, Feinleib M, McNamara PM, Castelli WP. Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. Circulation 1983; 67(5):968-977.

20 Kanhai DA, Kappelle LJ, van der Graaf Y, Uiterwaal CS, Visseren FL. The risk of general and abdominal adiposity in the occurrence of new vascular events and mortality in patients with various manifestations of vascular disease. Int J Obes (Lond) 2011.

21 Lakka HM, Lakka TA, Tuomilehto J, Salonen JT. Abdominal obesity is associated with increased risk of acute coronary events in men. Eur Heart J 2002; 23(9):706-713.

22 Sol BG, van der Graaf Y, Brouwer B, Hickox SM, Visseren FL. The effect of a self-management intervention to reduce vascular risk factors in patients with manifestations of vascular diseases. Eur J Cardiovasc Nurs 2010; 9(2):132-139.

23 de Jager SC, Kuiper J. Vaccination strategies in atherosclerosis. Thromb Haemost 2011; 106(5):796-803.

24 van Gils JM, Derby MC, Fernandes LR, Ramkhelawon B, Ray TD, Rayner KJ et al. The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting the emigration of macrophages from plaques. Nat Immunol 2012; 13(2):136-143.

25 Sacks HS, Fain JN. Human epicardial adipose tissue: a review. Am Heart J 2007; 153(6):907-917.

26 Miyata K, Shimokawa H, Kandabashi T, Higo T, Morishige K, Eto Y et al. Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. Arterioscler Thromb Vasc Biol 2000; 20(11):2351-2358.

27 Shimokawa H, Ito A, Fukumoto Y, Kadokami T, Nakaike R, Sakata M et al. Chronic treatment with interleukin-1 beta induces coronary intimal lesions and vasospastic responses in pigs in vivo. The role of platelet-derived growth factor. J Clin Invest 1996; 97(3):769-776.

28 Gao X, Mi S, Zhang F, Gong F, Lai Y, Gao F et al. Association of chemerin mRNA expression in human epicardial adipose tissue with coronary atherosclerosis. Cardiovasc Diabetol 2011; 10:87.

29 Iacobellis G, Pistilli D, Gucciardo M, Leonetti F, Miraldi F, Brancaccio G et al. Adiponectin expression in human epicardial adipose tissue in vivo is lower in patients with coronary artery disease. Cytokine 2005; 29(6):251-255.

30 Rutter MK, Meigs JB, Sullivan LM, D'Agostino RB, Sr., Wilson PW. Insulin resistance, the metabolic syndrome, and incident cardiovascular events in the Framingham Offspring Study. Diabetes 2005; 54(11):3252-3257.

31 Wassink AM, van der Graaf Y, van Haeften TW, Spiering W, Soedamah-Muthu SS, Visseren FL. Waist circumference and metabolic risk factors have separate and additive effects on the risk of future Type 2 diabetes in patients with vascular diseases. A cohort study. Diabet Med 2011; 28(8):932-940.

32 Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 2005; 112(17):2735-2752.

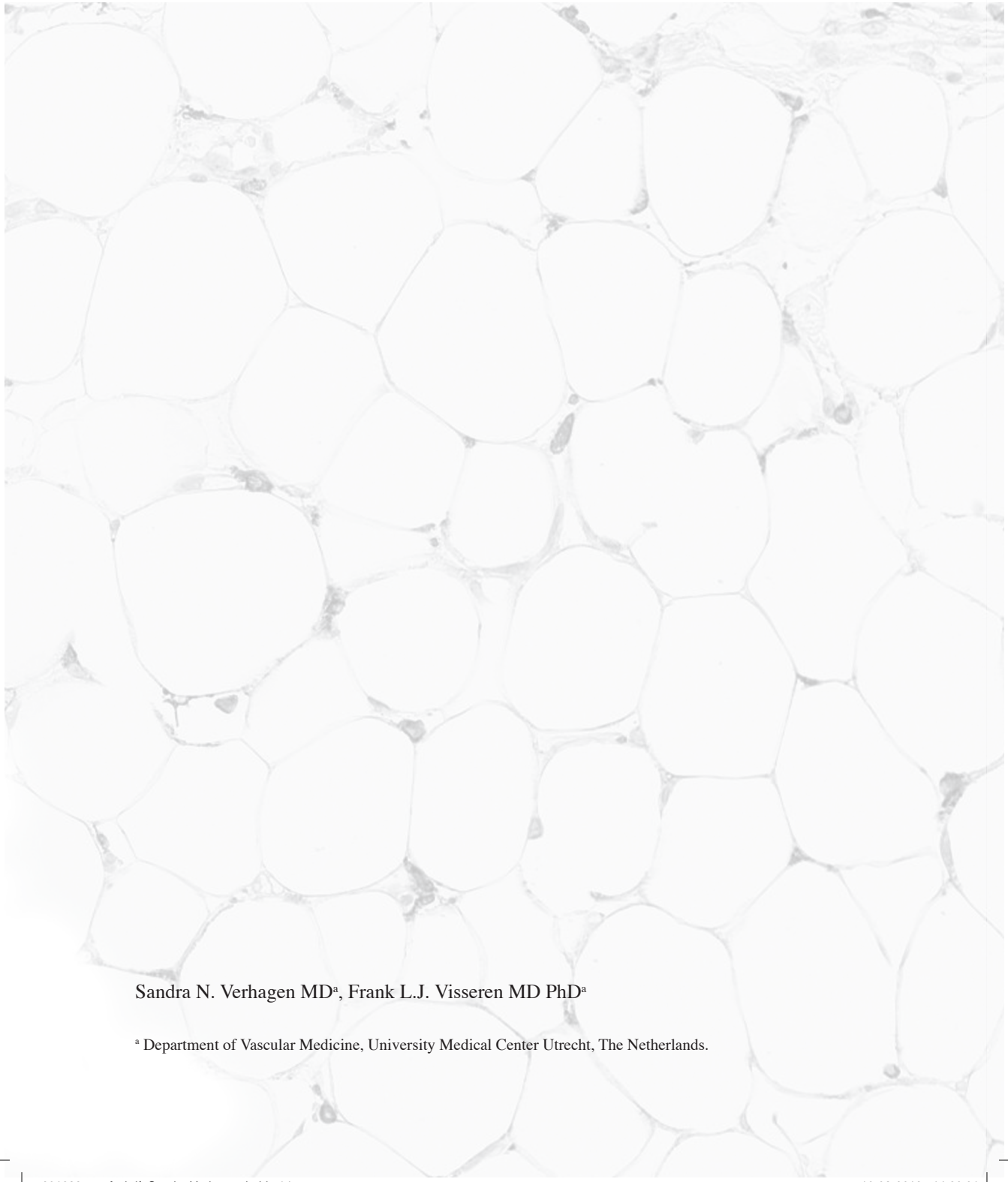
33 Tenenbaum A, Fisman EZ. "The metabolic syndrome... is dead": these reports are an exaggeration. Cardiovasc Diabetol 2011; 10(1):11.

34 Wassink AM, Olijhoek JK, Visseren FL. The metabolic syndrome: metabolic changes with vascular consequences. Eur J Clin Invest 2007; 37(1):8-17.

35 Mottillo S, Filion KB, Genest J, Joseph L, Pilote L, Poirier P et al. The metabolic syndrome and cardiovascular risk a systematic review and meta-analysis. J Am Coll Cardiol 2010; 56(14):1113-1132.

36 Wassink AM, van der Graaf Y, Olijhoek JK, Visseren FL. Metabolic syndrome and the risk of new vascular events and all-cause mortality in patients with coronary artery disease, cerebrovascular disease, peripheral arterial disease or abdominal aortic aneurysm. Eur Heart J 2008; 29(2):213-223.

37 Simons PC, Algra A, van de Laak MF, Grobbee DE, van der Graaf Y. Second manifestations of ARTerial disease (SMART) study: rationale and design. Eur J Epidemiol 1999; 15(9):773-781.



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Chapter 2

Perivascular adipose tissue as a cause of atherosclerosis

Atheroscerosis 2011; 214: 3-10.

ABSTRACT

Perivascular adipose tissue surrounds (coronary) arteries and may be involved in local stimulation of atherosclerotic plaque formation. Epicardial adipose tissue, the adipose tissue within the pericardium, is a frequently used measure of coronary perivascular adipose tissue and can be quantified with echocardiography, computed tomography (CT) and magnetic resonance imaging (MRI). The quantity of (coronary) perivascular adipose tissue is correlated with parameters of the metabolic syndrome, such as increased waist circumference, hypertriglyceridemia and hyperglycemia, and with coronary atherosclerosis. Coronary artery segments covered by myocardium are not exposed to coronary perivascular adipose tissue and interestingly, atherosclerosis is absent in these intramyocardial segments. Pro-inflammatory cytokines and adipokines are expressed and secreted at a higher level in epicardial adipose tissue of patients with coronary artery disease compared to patients without coronary artery disease. Furthermore, *in vitro* and *ex vivo* perivascular adipose tissue induces inflammation of the artery wall by secretion of pro-inflammatory proteins. Atherogenesis in the vascular wall is thus stimulated from 'outside-to-inside'. Based on the results of clinical, *ex vivo* and *in vitro* studies, it can be argued that perivascular adipose tissue may be involved in the process of atherosclerosis.

INTRODUCTION

Obesity, and in particular abdominal obesity, is associated with insulin resistance and atherosclerotic disease[1-3]. An increase in the visceral adipose tissue (VAT) compartment, as seen in abdominal adiposity, is accompanied by an increase in adipocyte size and adipose tissue dysfunction, resulting in augmented gene expression of inflammatory cytokines in adipocytes, infiltration of adipose tissue by macrophages and production of inflammatory cytokines by adipose tissue macrophages[4;5].
Most arteries are directly surrounded by adipose tissue, called perivascular adipose tissue. Both protective physiologic and pathologic properties of perivascular adipose tissue have been proposed[6]. Dysfunction or an excess of perivascular adipose tissue is thought to directly induce inflammation of the adjacent arteries and it can be hypothesized that perivascular adipose tissue may thus be involved in the pathogenesis of atherosclerosis, atherothrombosis and plaque rupture. In this concept initiation and progression of vascular diseases are mediated "from outside to inside"[7;8](figure 1).
In the present review we evaluate the evidence on the paracrine function and pathologic properties of perivascular adipose tissue on the vessel wall, based on *in vitro*, *ex vivo*, animal studies and clinical studies. We argue that perivascular adipose tissue is involved in the development of vascular diseases.

Defining adipose tissue depots

The term perivascular adipose tissue is used for adipose tissue around arteries irrespective of location. Epicardial adipose tissue is the adipose tissue around the heart reaching from the myocardium to the pericardium and accumulation is most prominent in the atrioventricular and interventricular grooves. Perivascular adipose tissue around coronary arteries is part of the epicardial adipose tissue compartment. There are no obvious anatomical boundaries between coronary perivascular adipose tissue and epicardial adipose tissue, although there may be functional differences. In subjects free of cardiovascular disease, the Macrophage Chemotactic Protein (MCP)-1 protein levels were higher in supernatants of coronary perivascular adipocytes compared to supernatants of epicardial adipocytes[9].
Besides epicardial adipose tissue, more adipose tissue is present in the mediastinum. The mediastinal adipose tissue outside of the pericardial sac is referred to as intra-thoracic adipose tissue (figure 2a). In the literature the term pericardial adipose tissue has been used for both epicardial adipose tissue and total mediastinal adipose tissue and should, in our view, not be used to describe epicardial adipose tissue. The epicardial and intra-thoracic adipose tissue depots are not supplied by the same vascular system. Epicardial adipose tissue is supplied by the coronary arteries themselves, intra-thoracic adipose tissue is supplied by the pericardiaphrenic artery, a branch of the internal thoracic artery[7].
Although adipocytes in coronary perivascular adipose tissue have the morphology of white adipose tissue, like VAT, they display reduced differentiation and maturation as compared to adipocytes in subcutaneous adipose tissue[9]. Also markers of brown adipose tissue are expressed in epicardial adipose tissue. PRDM16 and uncoupling protein-1 (UCP-1), markers of thermogenesis in brown adipose tissue are expressed at higher levels in coronary perivascular adipose tissue as compared to subcutaneous adipose tissue[10]. It was postulated that the brown adipose tissue properties are preventive against ventricular arrhythmias during drop of core temperature. However the significance of this matter remains to be elucidated.

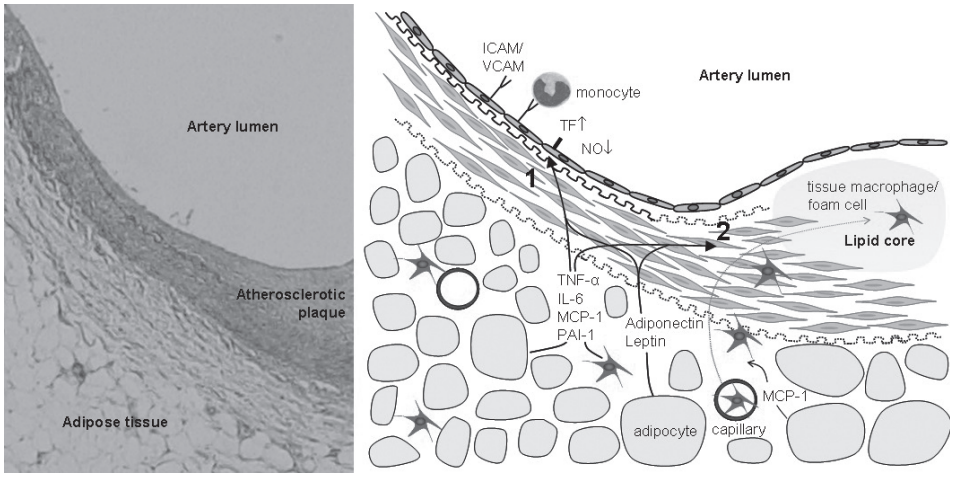
Imaging

Imaging techniques

Adipose tissue around (coronary) arteries has been quantified using various imaging techniques. Quantification of epicardial adipose tissue has been used in studies to evaluate the relation between adipose tissue around coronary arteries and atherosclerosis of the coronary arteries. With multi-slice computed tomography (CT) scanning it is possible to quantify adipose tissue, obtain information about coronary calcium and about coronary artery stenosis simultaneously[11].

Measurement of epicardial adipose tissue volume on CT is most often performed by tracing regions of interest on short axis views[12] (figure 2b). Adipose tissue voxels are usually identified as contiguous 3D voxels of -190 to -30 Houndsfield Units. Epicardial adipose tissue volume is then obtained by adding up the traced areas which are measured from the apex of the heart to the centre of the left atrium taking into account slice thickness and intersection gap between slices. Also automated computer assisted methods for quantification of epicardial and thoracic adipose tissue are available. When measuring using Qfat software five to seven control points are manually placed on the pericardium after which the pericardial contour is calculated and drawn[13;14]. Since these methods are only recently available, they are not used in all CT studies.

Figure 1 | Perivascular adipose tissue and its possible involvement in atherogenesis

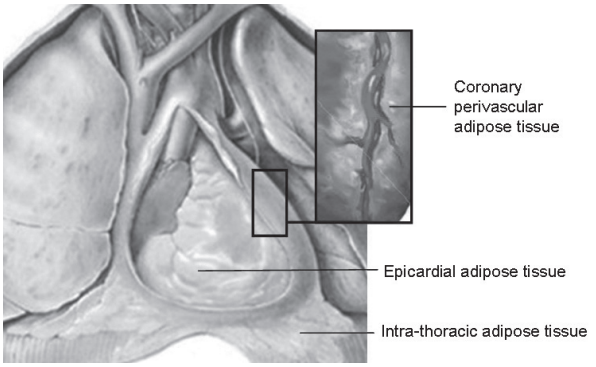


A. Histologic reproduction of the coronary artery and surrounding tissue. Perivascular adipose tissue is in close association with the artery wall which enables diffusion of adipokines and cytokines produced by the perivascular adipose tissue.

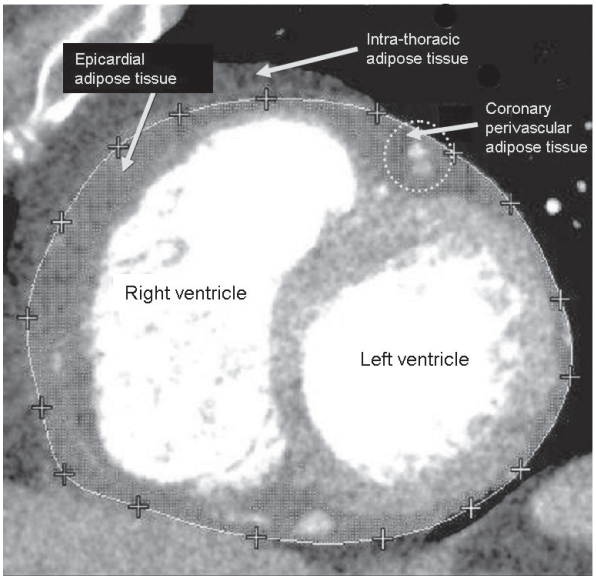
B. Model of the artery wall and perivascular adipose tissue. Adipocytes enlarge and increase the production of pro-inflammatory cytokines and adipokines. Cytokines such as TNF-alpha and IL-6 are also produced by macrophages in adipose tissue. Pro-inflammatory products of adipose tissue are able to diffuse to the surrounding tissues and structures. (1) Paracrine effects of cytokines and adipokines may be exerted on the endothelium causing endothelial dysfunction (decreased NO production), hypercoagulability (tissue factor and PAI-1 are upregulated), increased chemotaxis by upregulated MCP-1 and adhesion of monocytes to the endothelium by increased expression of adhesion molecules. (2) Also paracrine effects may be excerted directly on the adjacent tissues leading to influx of tissue macrophages into the artery wall from 'outside to inside' and to proliferation of smooth muscle cells.

See page 144 for a full color representation of this figure

Figure 2 | Nomenclature and quantification of adipose tissue within the mediastinum



A. Epicardial adipose tissue is defined as the adipose tissue within the pericardium. Intra-thoracic adipose tissue is defined as the adipose tissue within the mediastinum, but outside the pericardial sac. Perivascular adipose tissue is adipose tissue around arteries irrespective of location. Coronary perivascular adipose tissue is defined as the adipose tissue directly surrounding the coronary arteries.



B. Quantification of epicardial adipose tissue on CT image: Axial computed tomography image adapted from Gorter et al. Am. J. Cardiol. 2008[31]. Epicardial adipose tissue is measured by manually tracing the pericardium. Within the traced region, containing the heart and the epicardial adipose tissue, the adipose tissue is isolated by selecting a density range.

See page 145 for a full color representation of this figure

To compare adipose tissue quantity and atherosclerosis it is possible to measure adipose tissue around coronary arteries[12;15]. To avoid overestimation of the thickness, measurements are performed on axial views perpendicular to the surface of the heart. Mean coronary perivascular adipose tissue thickness in patients referred for diagnostic coronary angiography is 10.9±1.9 mm[12]. Despite the high spatial resolution of CT scanning, disadvantages like radiation exposition and high costs make it less practical for common use.

With transthoracic echocardiography epicardial adipose tissue thickness can easily be measured[16]. and it is frequently used in clinical studies[17-21]. Subjects are positioned in the left lateral decubitus position and the echocardiograms are recorded during several cardiac cycles. The epicardial adipose tissue is visible as an echo free space. Since the right ventricle is accessible from both parasternal long- and short axis views and contains the highest adipose tissue thickness, measurements of epicardial adipose tissue are made on the free wall of the right ventricle. The exact location of the

measurement on the free wall of the right ventricle is not fixed and epicardial adipose thickness is variable over this surface. This may be an explanation for the low intra- and interobserver correlations (0.63 (95%CI 0.40-0.77) and 0.61 (95%CI 0.37-0.75) respectively)[22], Although in that study reproducibility of the measurements was low, another study indicated a good reproducibility with a coefficient of variation between two sonographers of 3%[16].

Given the ability of CT to produce images with high spatial resolution and because atherosclerotic burden can be quantified in the same session, few studies have used MRI as a tool to measure epicardial adipose tissue. Similarly to CT, with MRI the contours of the epicardial adipose tissue are traced and adipose tissue voxels on slices are added up to calculate epicardial adipose tissue volume. There is a high correlation between epicardial adipose tissue on echocardiography and magnetic resonance imaging (MRI)[17]. Furthermore, inter-observer variability and reproducibility with MRI are higher for the volumetric approach compared to the one-dimensional approach of measuring epicardial adipose tissue thickness[23]. This is probably due to differences in epicardial adipose tissue thickness along the surface of the heart.

Determinants of epicardial adipose tissue quantity

The average amount of epicardial adipose tissue volume reported in population based groups ranges from 68±34 cm^3 to 124±50 cm^3 [24;25]. Like the VAT compartment, the epicardial adipose tissue compartment increases with age and is higher in men than in women[26-28]. Epicardial adipose tissue was reported to be 137±54cm^3 among men and 108± 41cm^3 among women of the Framingham offspring cohort[26]. In addition, epicardial adipose tissue quantity is strongly correlated to known risk factors for coronary artery disease (CAD) like visceral adiposity and parameters of the metabolic syndrome[17;29]. Especially VAT and waist circumference are highly correlated with epicardial adipose tissue quantity[16;30]. In patients with high BMI (>27 kg/m^2) epicardial adipose tissue volume was more than two times higher compared to those with a BMI <27 kg/m^2 (155±15 cm^3 versus 67±12 cm3)[31]. A strong correlation exists between fasting plasma glucose and epicardial adipose tissue measured with echocardiography or CT[29;32]. Epicardial adipose tissue quantity is higher in patients with type 2 diabetes compared to lean and obese patients without diabetes mellitus[28;33]. The difference in epicardial adipose tissue volume between men and women is even more pronounced in subjects with impaired fasting glucose and diabetes mellitus[28].

Epicardial and coronary perivascular adipose tissue and coronary atherosclerosis

The studies examining the relation between (coronary) perivascular adipose tissue quantity and atherosclerosis of the adjacent arteries are outlined in table 1. In the majority of studies increase of epicardial adipose tissue volume as well as coronary perivascular adipose tissue thickness were associated with stenosis of the coronary arteries. Since these studies are cross sectional studies it is uncertain whether adipose tissue plays a causal role in the development of atherosclerosis. It could also be argued that the causal relation between epicardial adipose tissue quantity and atherosclerosis of the adjacent vasculature is reverse. For instance, that an increase in adipose tissue volume is caused by atherosclerosis due to local vascular wall inflammation and post-ischemic changes. Importantly, two longitudinal studies have reported results that support the hypothesis of 'outside to inside signalling' as a cause of atherosclerosis[34;35]. Epicardial adipose tissue volume was measured and an increase of the quantity of epicardial adipose tissue was associated with incident coronary heart disease and with major adverse cardiac events. Associations were independent from BMI and other risk

Table 1 | Clinical studies investigating the relationship between adipose tissue quantity and atherosclerosis of the adjacent artery

Ref. no.	Patients	n	Determinant	Outcome measure	Association*
Echocardiography					
22	Referred for CAD	139	EAT thickness (mm)	CAD (stenosis ≥50% vs no significant stenosis)	EAT 2.22 ±1.86 vs 2.16 ± 1.77 p=0.83
24	Referred for CAD	203	EAT thickness >7.6mm	CAD (stenosis ≥50%)	OR 10.53 (95%CI 2.17-51.17)
21	Referred for CAD	527	EAT thickness ≥3mm	CAD (stenosis ≥50%)	OR 3.36 (95%CI 2.18-5.18)
23	Referred for CAD	150	EAT thickness ≥5.3mm	CAD (stenosis ≥20%)	OR 4.57 (95%CI 2.69-7.76)
52	Manifest arterial disease or high CAD risk	2726	Intra-abdominal fat (cm) (quartile 4 vs 1)	Infra-renal aortic diameter (mm)	β 1.38 (95%CI 0.72-2.04)
Computed tomography					
12	Referred for CAD	251	Pericardial fat volume per cm3	CAD (stenosis ≥50%)	-BMI<25: β 0.29 p<0.001 -BMI≥25: β 0.09 p<0.06
32	Referred for CAD	128	- EAT volume (cm3) - Pericoronary adipose tissue thickness (mm)	CAC (quartile 1 vs 4)	-BMI<27: EAT volume 69±10 vs 108±7 p=0.01; Thickness 8.2±0.5 vs 10.0±0.3 p=0.02 -BMI≥27: EATvolume 160±14 vs 115±10 p=0.1; Thickness 12.7±0.5 vs 11.1±0.4 p=0.1
45	Community based (no CAD)	1155	EAT volume per SD	CAC score >90th percentile of healthy referent sample	OR 1.27 (95%CI 1.06-1.52)
42	Referred for CAD	184	EAT volume >77ml	Coronary plaque (yes/no)	OR 1.03 (95%CI 1.01-1.05)
43	Healthy postmenopausal women	573	Pericoronary fat thickness (mm)	CAC score	β 0.16 (95%CI 0.02- 0.30)
13	Community based (no CAD)	398	EAT volume per SD	CAC score > 0	OR 1.38 (95%CI 1.04-1.84)

Ref. no.	Patients	n	Determinant	Outcome measure	Association*
36	Community based (no CAD)	998	EAT volume per SD	Cardiovascular event or death	HR 1.27 (95%CI 1.03-1.58)
46	Community based (no CAD)	159	EAT volume per SD	CAC score >0	OR 2.24 (95%CI 1.38-3.64)
41	Intermediate pre-test likelihood for CAD	264	Intra-thoracic fat volume >300ml	Coronary plaque (yes/no)	OR 4.1 (95%CI 3.6-4.3)
39	Referred for CAD	71	EAT volume ≥50cm3	CAD (total occlusion)	OR 4.64 (95%CI 1.21-17.72
47	Risk factors for CAD (no CAD)	201	EAT volume per log2 cm3	CAC score >0	OR 3.4 (95%CI 1.2-5.9)
14	Community based (including with CAD)	1267	EAT volume per SD	Clinical CAD (yes/no)	OR 1.92 (95%CI 1.23-3.02)
51	Peripheral arterial disease	148	Intra-abdominal fat ratio (tertile 3 vs 1)	aortic calcification (mm3) (tertile 3 vs 1)	OR 3.53 (95%CI 1.12-11.13)
38	Referred for CAD	171	EAT volume per SD	Coronary plaque (yes/no)	OR 2.9 (95%CI 1.6-5.1)
37	Referred for CAD	214	EAT volume >71cm3	Coronary plaque (yes/no)	OR 3.9 (95%CI 1.1-13.8)
42	Intermediate pre-test likelihood for CAD	200	EAT area (cm2)	Coronary plaque (no plaque vs plaque)	-BMI<25: 13.2±7.9 vs 18.7±11.5 p=0.006 -BMI≥25: 17.3±9.7 vs 17.4±8.4 p=0.991
53	Community based (no CAD)	1067	Perivascular adipose tissue of the thoracic aorta (cm3)	Aortic calcification (yes/no)	1.16 (95%CI 0.88-1.51)
18	Low pre-test likelihood for CAD	78	Coronary perivascular adipose tissue (cm3) (of 311 LAD segments)	Coronary plaque in the LAD (yes/no)	OR 3.15 (95%CI 2.20-4.51)
48	Community based (including with CAD)	1414	EAT volume + intrathoracic adipose tissue (cm3)	CAC score > 0	OR 1.34 (95%CI 1.10-1.64)
35	Patients with and without major cardiovascular events	58 vs 178	EAT volume cm3	Cardiovascular events after 4 years	OR 1.74 (95%CI 1.03-2.95)

Ref. no.: reference number; CAD: coronary artery disease; CAC: coronary artery calcium; EAT: epicardial adipose tissue; DM: diabetes mellitus; Coronary plaque: atherosclerotic plaque of the coronary arteries; OR: odds ratio with (95% confidence intervals); HR: hazard ratio with (95% confidence intervals)

* Reported odds ratios and betas are adjusted for a measure of adiposity and/ or other possible confounding variables. Reported absolute values are unadjusted.

factors, suggesting that epicardial adipose tissue is one of the factors contributing to CAD.
Compared to VAT, epicardial adipose tissue volume on CT had a stronger correlation with CAD in both obese and non-obese patients [11;25]. In contrast in one echocardiography study no associations between epicardial adipose tissue thickness and coronary artery stenosis could be demonstrated[19]. Differences in measuring epicardial adipose tissue may explain differences in reported associations given that standard deviations of measurements were wide. Possibly the high BMI (28.8+5.5 kg/m^2) of patients in that study is responsible for the lack of correlation, since the relation between epicardial adipose tissue quantity and CAD is reported to be modified by BMI[11;31]. Both total epicardial adipose tissue volume and coronary perivascular adipose tissue thickness were investigated and only in patients with low BMI (<27 kg/m^2 or <25 kg/m^2) a relation between epicardial adipose tissue and CAD was observed.
It has been investigated whether there is a link between epicardial adipose tissue and early stages of atherosclerosis and plaque vulnerability[36-38]. An association was demonstrated between epicardial adipose tissue and both non-stenotic lesions and non-calcified plaque independent of waist circumference[37]. Furthermore, epicardial adipose tissue volume is a strong and independent determinant of the presence of total coronary occlusions[37;38]. Since total coronary occlusion often originates from a ruptured instable plaque, it was hypothesized that epicardial adipose tissue volume is associated with plaque vulnerability[38]. Higher epicardial adipose tissue volume is seen in patients with non-calcified plaques than in patients with calcified plaques[36]. This may be relevant for the development of acute coronary syndromes as non-calcified parts of a plaque contribute to plaque vulnerability[39]. However this was not confirmed in other studies reporting equal epicardial adipose tissue volume in areas of calcified plaque and non-calcified plaque[37;40].
The association of epicardial adipose tissue and coronary atherosclerosis has been studied in patients with CAD or suspected of CAD[11;18-21;31;38;40-42], but also in groups of patients free of clinically evident CAD[24;25;43-46]. The relation between epicardial adipose tissue and coronary atherosclerosis is present in patients with clinical manifest CAD, in asymptomatic subjects, in subjects of Caucasian and Asian and possibly of Afro-American descent[47] and in patients with type 2 diabetes mellitus[33].
Given that epicardial adipose tissue quantity is highly correlated to risk factors of CAD[17;29;33;46], studies evaluating the causal relation between epicardial adipose tissue and CAD should be interpreted with caution. Especially adjustment for a parameter for VAT is necessary because VAT can act as a strong confounding variable in the relation between epicardial adipose tissue and CAD. VAT is associated with increased plasma concentrations of inflammatory markers[48]. Consequently adipose tissue dysfunction of VAT might accelerate atherogenesis of the coronary arteries in patients with high epicardial adipose tissue volume.

Relation between epicardial adipose tissue and other adipose tissue compartments
The intra-thoracic adipose tissue compartment is larger than the epicardial compartment and is therefore capable of secreting larger quantities of adipokines and cytokines. On the other hand, epicardial adipose tissue is anatomically closely related to coronary arteries and small quantities of adipokines may have large pathophysiological effects (figure 1). Congruent with the size of the depot, the relation between intra-thoracic adipose tissue volume and CAD was stronger than for epicardial adipose tissue volume and CAD in patients with risk factors for cardiovascular disease[46]. However, in the Framingham offspring study, comparable effect sizes of the total mediastinal adipose tissue (intra-thoracic plus epicardial adipose tissue) and epicardial adipose tissue compartments on

CAD were demonstrated[44]. The explanation of the comparable effects between depots may be caused by a relatively high pro-inflammatory state and relatively high production of adipokines and cytokines of perivascular adipose tissue[9]. Furthermore the short distance between the epicardial adipose tissue and the artery wall may enable paracrine signalling by direct diffusion of adipokines and cytokines[49] (figure 1).

There are relatively few studies evaluating the relation between the quantity of adipose tissue around arteries other than coronary arteries and atherosclerosis. The quantity of intra-abdominal adiposity, measured as waist circumference, with CT or ultrasound techniques, was associated with markers of aortic atherosclerosis[50;51]. Intra-thoracic adipose tissue was not correlated to calcification of the thoracic aorta although it was associated with calcification of coronary arteries and the abdominal aorta[52].

Adipokines and cytokines

Adipose tissue mainly consists of adipocytes and tissue macrophages and both cell types have secretory properties. Epicardial adipose tissue has the same embryologic origin as omental and mesenteric adipose tissue and is capable of producing a comparable pattern of cytokines and adipokines as VAT[53-55]. Secreted pro-inflammatory cytokines and adipokines may diffuse into the vessel wall to exert their (patho)physiological properties and may even enter the coronary circulation (figure 1)[56]. Usually, epicardial adipose tissue is sampled on the right ventricle and not proximal to the coronary arteries to study the endocrine profile *ex vivo*.

First adipokine and cytokine expression of epicardial adipose tissue harvested from patients with CAD and omental adipose tissue of patients without CAD was compared[57]. Expression of Plasminogen Activator Inhibitor (PAI)-1 and IL-6, was more pronounced in epicardial adipose tissue from CAD patients compared to omental adipose tissue from non-CAD patients and adiponectin expression less pronounced. No differences in expression of TNF-α, leptin and resistin were observed. In another study[58] the concentrations of TNF-α, IL-6, visfatin and leptin in supernatants of incubated epicardial adipose tissue from patients with CAD were higher than in supernatants from patients without CAD. Adiponectin plasma concentrations were 0.41±0.31 ng/l in patients with CAD and more than ten times higher in patients without CAD[58]. Analogously lower epicardial adipose tissue adiponectin protein expression was observed in patients with CAD compared to patients without CAD in a small sample of 16 patients[59]. Furthermore the extent of CAD, expressed as number of injured arteries is associated with IL-6 plasma concentration and inversely associated with adiponectin gene expression in epicardial adipose tissue[60]. In post mortem samples, expression of adipokines and cytokines in perivascular adipose tissue of the aorta and coronary arteries was found to be correlated to atherosclerosis, whereas expression in epicardial adipose tissue was not[61]. Apparently, coronary perivascular adipose tissue is more metabolically active than epicardial adipose tissue.

Recently more proteins expressed by epicardial adipose tissue were identified. Fatty acid binding protein 4 is expressed in epicardial adipose tissue and levels of expression are increased in the metabolic syndrome[62]. Plasma adrenomedulin is compensatory elevated after cardiovascular events and patients with CAD have higher epicardial adipose tissue expression of this protein than patients without CAD[63]. With microchip array 271 genes encoding proteins overexpressed in epicardial adipose tissue as opposed to subcutaneous adipose tissue were identified[64]. Out of these 271 genes, secretory type 2 phospholipase A2 was shown to have the highest overexpression. Furthermore

expression and secretion was higher in CAD patients as compared to patients free of CAD.
In addition to local pro-atherosclerotic effects it has been suggested that epicardial adipose tissue plays a role in the pathogenesis of hypertension and insulin resistance[65;66]. However since the analyses in these studies were not adjusted for VAT it remains unclear whether this small adipose tissue depot actually induces systemic effects such as hypertension or insulin resistance. Also expression of adiponectin and leptin in epicardial and subcutaneous adipose tissue is comparable in patients with and without diabetes[67].
In studies investigating the expression levels of cytokines and adipokines it should be taken into account that the time of biopsy during surgery influences the absolute levels of adipokine and cytokine production by epicardial adipose tissue[68]. It has been demonstrated that IL-6, TNF-α, MCP-1, leptin and resistin levels are expressed at a higher level in epicardial adipose tissue at the end of cardiac surgery compared to the start of surgery in contrast to adiponectin levels that were not significantly affected.
In patients undergoing percutaneous coronary intervention for acute myocardial infarction increased levels of serum amyloid A (SAA) and IL-6 and decreased levels of C-reactive protein (CRP) were found near the lesion compared to an upstream location. This demonstrates cytokine production by the atherosclerotic plaque which in turn may influence the surrounding perivascular adipose tissue[69]. Therefore it can be hypothesized that there is a vicious cycle wherein perivascular adipose tissue and atherosclerotic lesions augment each others pro-inflammatory state by paracrine signaling.

Arteries protected against adipose tissue products

An argument for a causal relation between perivascular adipose tissue and atherosclerosis would be if absence of adipose tissue around coronary artery segments was related to absence of atherosclerosis in that location. This is demonstrated by absence of atherosclerosis in coronary artery segments with myocardial bridges[70]. A myocardial bridge is a variant of coronary artery anatomy where part of the coronary artery has an intra-myocardial course. Therefore at sites of myocardial bridges coronary artery segments are covered with myocardium and not surrounded by adipose tissue. In post mortem studies as well as in studies using CT, coronary arteries proximal to myocardial bridges showed increased atherosclerosis whereas the segments with overlying myocardial bridge were free of atherosclerosis[71-75]. Proximal to the myocardial bridge there is more atherosclerosis which is even more abundant when the myocardial bridge is longer and thicker, possibly due to haemostatic factors[72;73;76]. It was suspected that an increase in shear stress at the location of a myocardial bridge decreases the adhesion of monocytes to the endothelium and decreased lipid transfer across the artery wall[76;77]. In a study using electron microscopy this hypothesis was confirmed[71]. A spindle shape of endothelial cells was demonstrated in the bridged segment indicating high shear stress compared to a more round shape at the epicardial segment indicating low shear stress. In our view, the lack of surrounding adipose tissue and therefore absence of pro-inflammatory stimuli is responsible for the athero-protective property of the myocardial bridge. However, in patients with lipodystrophy atherosclerotic changes of the coronary arteries were shown post mortem despite scant epicardial adipose tissue and VAT[71;78].
On the other hand, the myocardial side of the coronary artery is relatively protected against influences from epicardial adipose tissue. Plaque coordination within a vessel was studied with intravascular ultrasound and more plaques showed a epicardial distribution[79]. The preference for an epicardial distribution of atherosclerotic lesions implicates a role for involvement of tissue from outside the

vessel wall, such as adipose tissue, or for mechanical forces in plaque formation.

Outside to inside signaling in atherosclerosis

Involvement of the adventitial side of the vessel wall in atherosclerosis has already been proposed in 1989, by the observation of leucocyte migration into the vessel wall from outside[80]. In that study, endotoxin soaked cotton was implanted on the ventral side of rat femoral arteries. Only on the endotoxin side leukocyte migration and smooth muscle cell rich intimal lesions occurred. Leukocytes migrated from both the luminal and the adventitial side. Further in vivo experiments in pigs have shown that external application of the inflammatory cytokines IL-1β or MCP-1 on pig coronary arteries induced an increase in intima thickness and arterial remodeling in vivo[81;82]. In the case of MCP-1 application, invasion of adventitia-derived macrophages into the vascular wall was observed. This process was mediated by Rho-kinase.

The cytokines MCP-1 and IL-8 were detected in the supernatants of human perivascular adipose tissue of the aorta and these supernatants caused migration of granulocytes, monocytes and IL-1 activated T-cells in vitro[83]. Pre-incubation with anti-MCP-1 and anti-IL-8 inhibited the migration of peripheral blood leucocytes. Furthermore conditioned medium of epicardial adipose tissue is able to increase migration of human monocytes and adhesion on human endothelial cells[84]. This demonstrates that perivascular adipose tissue has properties that may influence the process of vascular wall inflammation, an important step in atherogenesis.

On the contrary, perivascular adipose tissue also had protective effects on vascular tone. In rat mesenteric artery or aorta rings the presence of perivascular adipose tissue was associated with reduced dose-dependent response on vasoconstricting agents[85;86]. In human small arteries this effect was observed as well, although in obese subjects with the metabolic syndrome there was a loss of vasodilator effect of adipose tissue[87]. Furthermore, in coronary arteries of lean dogs the presence of perivascular adipose tissue diminished the vasodilating effect of bradykinin[88]. This was mediated via protein kinase C-β phosphorilation of nitric oxide (NO) synthase. Perivascular adipose tissue has no effect on smooth muscle response to NO, superoxide (O_2-)production or hydrogen peroxide (H_2O_2) mediated vasodilatation[89].

Recently macrophage infiltration and cytokine expression in perivascular adipose tissue of the aorta was studied in a murine model using angiotensin 2 to induce aortic aneurysm[90]. Obesity induced increased cytokine expression and macrophage infiltration in perivascular adipose tissue of the aorta and resulted in an increase in aortic diameter. From this observation it may be concluded that in mice obesity catalyses the atherosclerotic process and that adipose tissue dysfunction might be an important causative factor.

In conclusion

Adipose tissue directly surrounding (coronary) arteries has inflammatory properties and the quantity of perivascular adipose tissue is related to the presence of atherosclerosis. Results from *in vitro*, *ex vivo*, animal and clinical studies support the hypothesis that perivascular adipose tissue is able to influence the atherosclerotic process in the artery wall 'from outside to inside'. However, it should be noted that adipose tissue around (coronary) arteries is correlated with visceral adipose tissue and thus with systemic metabolic changes associated with obesity. Therefore it remains to be elucidated whether perivascular adipose tissue is independently involved in atherogenesis.

REFERENCE LIST

1 Janssen I, Katzmarzyk PT, Ross R. Waist circumference and not body mass index explains obesity-related health risk. Am.J.Clin.Nutr. 2004; 79(3): 379-84.

2 Rimm EB, Stampfer MJ, Giovannucci E, Ascherio A, Spiegelman D, Colditz GA, Willett WC. Body size and fat distribution as predictors of coronary heart disease among middle-aged and older US men. Am.J.Epidemiol. 1995; 141(12): 1117-27.

3 Yusuf S, Hawken S, Ounpuu S, Bautista L, Franzosi MG, Commerford P, Lang CC, Rumboldt Z, Onen CL, Lisheng L, Tanomsup S, Wangai P, Jr., Razak F, Sharma AM, Anand SS. Obesity and the risk of myocardial infarction in 27,000 participants from 52 countries: a case-control study. Lancet 2005; 366(9497): 1640-9.

4 Maffeis C, Silvagni D, Bonadonna R, Grezzani A, Banzato C, Tato L. Fat cell size, insulin sensitivity, and inflammation in obese children. J.Pediatr. 2007; 151(6): 647-52.

5 Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. Diabetologia 2000; 43(12): 1498-506.

6 Iacobellis G, Gao YJ, Sharma AM. Do cardiac and perivascular adipose tissue play a role in atherosclerosis? Curr.Diab.Rep. 2008; 8(1): 20-4.

7 Sacks HS, Fain JN. Human epicardial adipose tissue: a review. Am Heart J. 2007; 153(6): 907-17.

8 Stern N, Marcus Y. Perivascular fat: innocent bystander or active player in vascular disease? J.Cardiometab. Syndr. 2006; 1(2): 115-20.

9 Chatterjee TK, Stoll LL, Denning GM, Harrelson A, Blomkalns AL, Idelman G, Rothenberg FG, Neltner B, Romig-Martin SA, Dickson EW, Rudich S, Weintraub NL. Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. Circ.Res. 2009; 104(4): 541-9.

10 Sacks HS, Fain JN, Holman B, Cheema P, Chary A, Parks F, Karas J, Optican R, Bahouth SW, Garrett E, Wolf RY, Carter RA, Robbins T, Wolford D, Samaha J. Uncoupling Protein-1 and related mRNAs in Human Epicardial and Other Adipose Tissues: Epicardial Fat functioning as Brown Fat. J.Clin.Endocrinol. Metab 2009.

11 Taguchi R, Takasu J, Itani Y, Yamamoto R, Yokoyama K, Watanabe S, Masuda Y. Pericardial fat accumulation in men as a risk factor for coronary artery disease. Atherosclerosis 2001; 157(1): 203-9.

12 Gorter PM, van Lindert AS, de Vos AM, Meijs MF, van der Graaf Y, Doevendans PA, Prokop M, Visseren FL. Quantification of epicardial and peri-coronary fat using cardiac computed tomography; reproducibility and relation with obesity and metabolic syndrome in patients suspected of coronary artery disease. Atherosclerosis 2008; 197(2): 896-903.

13 Bandekar AN, Naghavi M, Kakadiaris IA. Automated pericardial fat quantification in CT data. Conf.Proc. IEEE Eng Med.Biol.Soc. 2006; 1, 932-5.

14 Dey D, Suzuki Y, Suzuki S, Ohba M, Slomka PJ, Polk D, Shaw LJ, Berman DS. Automated quantitation of pericardiac fat from noncontrast CT. Invest Radiol. 2008; 43(2): 145-53.

15 Mahabadi AA, Reinsch N, Lehmann N, Altenbernd J, Kalsch H, Seibel RM, Erbel R, Mohlenkamp S. Association of pericoronary fat volume with atherosclerotic plaque burden in the underlying coronary artery: A segment analysis. Atherosclerosis 2010.

16 Iacobellis G, Assael F, Ribaudo MC, Zappaterreno A, Alessi G, Di MU, Leonetti F. Epicardial fat from echocardiography: a new method for visceral adipose tissue prediction. Obes.Res. 2003; 11(2): 304-10.

17 Iacobellis G, Ribaudo MC, Assael F, Vecci E, Tiberti C, Zappaterreno A, Di MU, Leonetti F. Echocardiographic epicardial adipose tissue is related to anthropometric and clinical parameters of metabolic syndrome: a new indicator of cardiovascular risk. J.Clin.Endocrinol.Metab 2003; 88(11): 5163-8.

18 Ahn SG, Lim HS, Joe DY, Kang SJ, Choi BJ, Choi SY, Yoon MH, Hwang GS, Tahk SJ, Shin JH. Relationship of epicardial adipose tissue by echocardiography to coronary artery disease. Heart 2008; 94(3): e7.

19 Chaowalit N, Somers VK, Pellikka PA, Rihal CS, Lopez-Jimenez F. Subepicardial adipose tissue and the presence and severity of coronary artery disease. Atherosclerosis 2006; 186(2): 354-9.

20 Eroglu S, Sade LE, Yildirir A, Bal U, Ozbicer S, Ozgul AS, Bozbas H, Aydinalp A, Muderrisoglu H. Epicardial adipose tissue thickness by echocardiography is a marker for the presence and severity of coronary artery disease. Nutr.Metab Cardiovasc.Dis. 2008.

21 Jeong JW, Jeong MH, Yun KH, Oh SK, Park EM, Kim YK, Rhee SJ, Lee EM, Lee J, Yoo NJ, Kim NH, Park JC. Echocardiographic epicardial fat thickness and coronary artery disease. Circ.J. 2007; 71(4): 536-9.

22 Saura D, Oliva MJ, Rodriguez D, Pascual-Figal DA, Hurtado JA, Pinar E, de la MG, Valdes M. Reproducibility of echocardiographic measurements of epicardial fat thickness. Int.J.Cardiol. 2008.

23 Fluchter S, Haghi D, Dinter D, Heberlein W, Kuhl HP, Neff W, Sueselbeck T, Borggrefe M, Papavassiliu T. Volumetric assessment of epicardial adipose tissue with cardiovascular magnetic resonance imaging. Obesity.(Silver.Spring) 2007; 15(4): 870-8.

24 Ding J, Kritchevsky SB, Hsu FC, Harris TB, Burke GL, Detrano RC, Szklo M, Criqui MH, Allison M, Ouyang P, Brown ER, Carr JJ. Association between non-subcutaneous adiposity and calcified coronary plaque: a substudy of the Multi-Ethnic Study of Atherosclerosis. Am.J.Clin.Nutr. 2008; 88(3): 645-50.

25 Mahabadi AA, Massaro JM, Rosito GA, Levy D, Murabito JM, Wolf PA, O'Donnell CJ, Fox CS, Hoffmann U. Association of pericardial fat, intrathoracic fat, and visceral abdominal fat with cardiovascular disease burden: the Framingham Heart Study. Eur.Heart J. 2009; 30(7): 850-6.

26 Fox CS, Gona P, Hoffmann U, Porter SA, Salton CJ, Massaro JM, Levy D, Larson MG, D'Agostino RB, Sr., O'Donnell CJ, Manning WJ. Pericardial fat, intrathoracic fat, and measures of left ventricular structure and function: the Framingham Heart Study. Circulation 2009; 119(12): 1586-91.

27 Silaghi A, Piercecchi-Marti MD, Grino M, Leonetti G, Alessi MC, Clement K, Dadoun F, Dutour A. Epicardial adipose tissue extent: relationship with age, body fat distribution, and coronaropathy. Obesity. (Silver.Spring) 2008; 16(11): 2424-30.

28 Iozzo P, Lautamaki R, Borra R, Lehto HR, Bucci M, Viljanen A, Parkka J, Lepomaki V, Maggio R, Parkkola R, Knuuti J, Nuutila P. Contribution of glucose tolerance and gender to cardiac adiposity. J.Clin. Endocrinol.Metab 2009; 94(11): 4472-82.

29 Wang TD, Lee WJ, Shih FY, Huang CH, Chang YC, Chen WJ, Lee YT, Chen MF. Relations of epicardial adipose tissue measured by multidetector computed tomography to components of the metabolic syndrome are region-specific and independent of anthropometric indexes and intraabdominal visceral fat. J.Clin. Endocrinol.Metab 2009; 94(2): 662-9.

30 Wheeler GL, Shi R, Beck SR, Langefeld CD, Lenchik L, Wagenknecht LE, Freedman BI, Rich SS, Bowden DW, Chen MY, Carr JJ. Pericardial and visceral adipose tissues measured volumetrically with computed tomography are highly associated in type 2 diabetic families. Invest Radiol. 2005; 40(2): 97-101.

31 Gorter PM, de Vos AM, van der Graaf Y, Stella PR, Doevendans PA, Meijs MF, Prokop M, Visseren FL. Relation of epicardial and pericoronary fat to coronary atherosclerosis and coronary artery calcium in patients undergoing coronary angiography. Am.J.Cardiol. 2008; 102(4): 380-5.

32 Iacobellis G, Leonetti F. Epicardial adipose tissue and insulin resistance in obese subjects. J.Clin. Endocrinol.Metab 2005; 90(11): 6300-2.

33 Wang CP, Hsu HL, Hung WC, Yu TH, Chen YH, Chiu CA, Lu LF, Chung FM, Shin SJ, Lee YJ. Increased epicardial adipose tissue (EAT) volume in type 2 diabetes mellitus and association with metabolic syndrome and severity of coronary atherosclerosis. Clin.Endocrinol.(Oxf) 2009; 70(6): 876-82.

34 Cheng VY, Dey D, Tamarappoo B, Nakazato R, Gransar H, Miranda-Peats R, Ramesh A, Wong ND, Shaw LJ, Slomka PJ, Berman DS. Pericardial fat burden on ECG-gated noncontrast CT in asymptomatic patients who subsequently experience adverse cardiovascular events. JACC.Cardiovasc.Imaging 2010; 3(4): 352-60.

35 Ding J, Hsu FC, Harris TB, Liu Y, Kritchevsky SB, Szklo M, Ouyang P, Espeland MA, Lohman KK, Criqui MH, Allison M, Bluemke DA, Carr JJ. The association of pericardial fat with incident coronary heart disease: the Multi-Ethnic Study of Atherosclerosis (MESA). Am.J.Clin.Nutr. 2009; 90(3): 499-504.

36 Alexopoulos N, McLean DS, Janik M, Arepalli CD, Stillman AE, Raggi P. Epicardial adipose tissue and coronary artery plaque characteristics. Atherosclerosis 2009.

37 Konishi M, Sugiyama S, Sugamura K, Nozaki T, Ohba K, Matsubara J, Matsuzawa Y, Sumida H, Nagayoshi Y, Nakaura T, Awai K, Yamashita Y, Jinnouchi H, Matsui K, Kimura K, Umemura S, Ogawa H. Association of pericardial fat accumulation rather than abdominal obesity with coronary atherosclerotic plaque formation in patients with suspected coronary artery disease. Atherosclerosis 2009.

38 Ueno K, Anzai T, Jinzaki M, Yamada M, Jo Y, Maekawa Y, Kawamura A, Yoshikawa T, Tanami Y, Sato K, Kuribayashi S, Ogawa S. Increased Epicardial Fat Volume Quantified by 64-Multidetector Computed Tomography is Associated With Coronary Atherosclerosis and Totally Occlusive Lesions. Circ.J. 2009.

39 Pundziute G, Schuijf JD, Jukema JW, Decramer I, Sarno G, Vanhoenacker PK, Boersma E, Reiber JH, Schalij MJ, Wijns W, Bax JJ. Evaluation of plaque characteristics in acute coronary syndromes: non-invasive assessment with multi-slice computed tomography and invasive evaluation with intravascular ultrasound radiofrequency data analysis. Eur.Heart J. 2008; 29(19): 2373-81.

40 Greif M, Becker A, von ZF, Lebherz C, Lehrke M, Broedl UC, Tittus J, Parhofer K, Becker C, Reiser M, Knez A, Leber AW. Pericardial adipose tissue determined by dual source CT is a risk factor for coronary atherosclerosis. Arterioscler.Thromb.Vasc.Biol. 2009; 29(5): 781-6.

41 Djaberi R, Schuijf JD, van Werkhoven JM, Nucifora G, Jukema JW, Bax JJ. Relation of epicardial adipose tissue to coronary atherosclerosis. Am.J.Cardiol. 2008; 102(12): 1602-7.

42 Yong HS, Kim EJ, Seo HS, Kang EY, Kim YK, Woo OH, Han H. Pericardial fat is more abundant in patients with coronary atherosclerosis and even in the non-obese patients: evaluation with cardiac CT angiography. Int.J.Cardiovasc.Imaging 2009.

43 de Vos AM, Prokop M, Roos CJ, Meijs MF, van der Schouw YT, Rutten A, Gorter PM, Cramer MJ, Doevendans PA, Rensing BJ, Bartelink ML, Velthuis BK, Mosterd A, Bots ML. Peri-coronary epicardial adipose tissue is related to cardiovascular risk factors and coronary artery calcification in post-menopausal women. Eur.Heart J. 2008; 29(6): 777-83.

44 Rosito GA, Massaro JM, Hoffmann U, Ruberg FL, Mahabadi AA, Vasan RS, O'Donnell CJ, Fox CS. Pericardial fat, visceral abdominal fat, cardiovascular disease risk factors, and vascular calcification in a community-based sample: the Framingham Heart Study. Circulation 2008; 117(5): 605-13.

45 Ding J, Kritchevsky SB, Harris TB, Burke GL, Detrano RC, Szklo M, Jeffrey CJ, Multi-Ethnic Study of Atherosclerosis. The association of pericardial fat with calcified coronary plaque. Obesity.(Silver.Spring) 2008; 16(8): 1914-9.

46 Dey D, Wong ND, Tamarappoo B, Nakazato R, Gransar H, Cheng VY, Ramesh A, Kakadiaris I, Germano G, Slomka PJ, Berman DS. Computer-aided non-contrast CT-based quantification of pericardial and thoracic fat and their associations with coronary calcium and metabolic syndrome. Atherosclerosis 2009.

47 Liu J, Fox CS, Hickson D, Sarpong D, Ekunwe L, May WD, Hundley GW, Carr JJ, Taylor HA. Pericardial Adipose tissue, Atherosclerosis and Cardiovascular Disease Risk Factors: the Jackson Heart Study. Diabetes Care 2010.

48 Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG, Keaney JF, Jr., Meigs JB, Lipinska I, Kathiresan S, Murabito JM, O'Donnell CJ, Benjamin EJ, Fox CS. Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham Heart Study. Circulation 2007; 116(11): 1234-41.

49 Thalmann S, Meier CA. Local adipose tissue depots as cardiovascular risk factors. Cardiovasc.Res. 2007; 75(4): 690-701.

50 Golledge J, Jayalath R, Oliver L, Parr A, Schurgers L, Clancy P. Relationship between CT anthropometric measurements, adipokines and abdominal aortic calcification. Atherosclerosis 2008; 197(1): 428-34.

51 Gorter PM, Visseren FL, Moll FL, van der Graaf Y. Intra-abdominal fat and metabolic syndrome are associated with larger infrarenal aortic diameters in patients with clinically evident arterial disease. J.Vasc. Surg. 2008; 48(1): 114-20.

52 Lehman SJ, Massaro JM, Schlett CL, O'Donnell CJ, Hoffmann U, Fox CS. Peri-aortic fat, cardiovascular disease risk factors, and aortic calcification: The Framingham Heart Study. Atherosclerosis 2010.

53 Fain JN, Sacks HS, Bahouth SW, Tichansky DS, Madan AK, Cheema PS. Human epicardial adipokine messenger RNAs: comparisons of their expression in substernal, subcutaneous, and omental fat. Metabolism 2010.

54 Ho E, Shimada Y. Formation of the epicardium studied with the scanning electron microscope. Dev.Biol. 1978; 66(2): 579-85.

55 Mazurek T, Zhang L, Zalewski A, Mannion JD, Diehl JT, Arafat H, Sarov-Blat L, O'Brien S, Keiper EA, Johnson AG, Martin J, Goldstein BJ, Shi Y. Human epicardial adipose tissue is a source of inflammatory mediators. Circulation 2003; 108(20): 2460-6.

56 Tadros TM, Massaro JM, Rosito GA, Hoffmann U, Vasan RS, Larson MG, Keaney Jr JF, Lipinska I, Meigs JB, Kathiresan S, O'Donnell CJ, Fox CS, Benjamin EJ. Pericardial Fat Volume Correlates With Inflammatory Markers: The Framingham Heart Study. Obesity.(Silver.Spring) 2009.

57 Baker AR, Silva NF, Quinn DW, Harte AL, Pagano D, Bonser RS, Kumar S, McTernan PG. Human epicardial adipose tissue expresses a pathogenic profile of adipocytokines in patients with cardiovascular disease. Cardiovasc.Diabetol. 2006; 5, 1.

58 Cheng KH, Chu CS, Lee KT, Lin TH, Hsieh CC, Chiu CC, Voon WC, Sheu SH, Lai WT. Adipocytokines and proinflammatory mediators from abdominal and epicardial adipose tissue in patients with coronary artery disease. Int.J.Obes.(Lond) 2008; 32(2): 268-74.

59 Iacobellis G, Pistilli D, Gucciardo M, Leonetti F, Miraldi F, Brancaccio G, Gallo P, di Gioia CR. Adiponectin expression in human epicardial adipose tissue in vivo is lower in patients with coronary artery disease. Cytokine 2005; 29(6): 251-5.

60 Eiras S, Teijeira-Fernandez E, Shamagian LG, Fernandez AL, Vazquez-Boquete A, Gonzalez-Juanatey JR. Extension of coronary artery disease is associated with increased IL-6 and decreased adiponectin gene expression in epicardial adipose tissue. Cytokine 2008; 43(2): 174-80.

61 Spiroglou SG, Kostopoulos CG, Varakis JN, Papadaki HH. Adipokines in periaortic and epicardial adipose tissue: differential expression and relation to atherosclerosis. J.Atheroscler.Thromb. 2010; 17(2): 115-30.

62 Vural B, Atalar F, Ciftci C, Demirkan A, Susleyici-Duman B, Gunay D, Akpinar B, Sagbas E, Ozbek U, Buyukdevrim AS. Presence of fatty-acid-binding protein 4 expression in human epicardial adipose tissue in metabolic syndrome. Cardiovasc.Pathol. 2008; 17(6): 392-8.

63 Silaghi A, Achard V, Paulmyer-Lacroix O, Scridon T, Tassistro V, Duncea I, Clement K, Dutour A, Grino M. Expression of adrenomedullin in human epicardial adipose tissue: role of coronary status. Am.J.Physiol Endocrinol.Metab 2007; 293(5): E1443-E1450.

64 Dutour A, Achard V, Sell H, Naour N, Collart F, Gaborit B, Silaghi A, Eckel J, Alessi MC, Henegar C, Clement K. Secretory Type II Phospholipase A2 Is Produced and Secreted by Epicardial Adipose Tissue and Overexpressed in Patients with Coronary Artery Disease. J.Clin.Endocrinol.Metab 2009.

65 Roubicek T, Dolinkova M, Blaha J, Haluzikova D, Bosanska L, Mraz M, Kremen J, Haluzik M. Increased angiotensinogen production in epicardial adipose tissue during cardiac surgery: possible role in a postoperative insulin resistance. Physiol Res. 2008; 57(6): 911-7.

66 Teijeira-Fernandez E, Eiras S, Grigorian-Shamagian L, Fernandez A, Adrio B, Gonzalez-Juanatey JR. Epicardial adipose tissue expression of adiponectin is lower in patients with hypertension. J.Hum. Hypertens. 2008; 22(12): 856-63.

67 Teijeira-Fernandez E, Eiras S, Grigorian-Shamagian L, Salgado-Somoza A, Martinez-Comendador JM, Gonzalez-Juanatey JR. Diabetic and nondiabetic patients express similar adipose tissue adiponectin and leptin levels. Int.J.Obes.(Lond) 2010.

68 Kremen J, Dolinkova M, Krajickova J, Blaha J, Anderlova K, Lacinova Z, Haluzikova D, Bosanska L, Vokurka M, Svacina S, Haluzik M. Increased subcutaneous and epicardial adipose tissue production of proinflammatory cytokines in cardiac surgery patients: possible role in postoperative insulin resistance. J.Clin.Endocrinol.Metab 2006; 91(11): 4620-7.

69 Maier W, Altwegg LA, Corti R, Gay S, Hersberger M, Maly FE, Sutsch G, Roffi M, Neidhart M, Eberli FR, Tanner FC, Gobbi S, von EA, Luscher TF. Inflammatory markers at the site of ruptured plaque in acute myocardial infarction: locally increased interleukin-6 and serum amyloid A but decreased C-reactive protein. Circulation 2005; 111(11): 1355-61.

70 Lee SS, Wu TL. The role of the mural coronary artery in prevention of coronary atherosclerosis. Arch. Pathol. 1972; 93(1): 32-5.

71 Ishii T, Asuwa N, Masuda S, Ishikawa Y. The effects of a myocardial bridge on coronary atherosclerosis and ischaemia. J.Pathol. 1998; 185(1): 4-9.

72 Ishikawa Y, Akasaka Y, Ito K, Akishima Y, Kimura M, Kiguchi H, Fujimoto A, Ishii T. Significance of anatomical properties of myocardial bridge on atherosclerosis evolution in the left anterior descending coronary artery. Atherosclerosis 2006; 186(2): 380-9.

73 Ishikawa Y, Akasaka Y, Suzuki K, Fujiwara M, Ogawa T, Yamazaki K, Niino H, Tanaka M, Ogata K, Morinaga S, Ebihara Y, Kawahara Y, Sugiura H, Takimoto T, Komatsu A, Shinagawa T, Taki K, Satoh H, Yamada K, Yanagida-Iida M, Shimokawa R, Shimada K, Nishimura C, Ito K, Ishii T. Anatomic properties of myocardial bridge predisposing to myocardial infarction. Circulation 2009; 120(5): 376-83.

74 Kawawa Y, Ishikawa Y, Gomi T, Nagamoto M, Terada H, Ishii T, Kohda E. Detection of myocardial bridge and evaluation of its anatomical properties by coronary multislice spiral computed tomography. Eur.J.Radiol. 2007; 61(1): 130-8.

75 Zeina AR, Odeh M, Blinder J, Rosenschein U, Barmeir E. Myocardial bridge: evaluation on MDCT. AJR Am.J.Roentgenol. 2007; 188(4): 1069-73.

76 Scher AM. Absence of atherosclerosis in human intramyocardial coronary arteries: a neglected phenomenon. Atherosclerosis 2000; 149(1): 1-3.

77 Ishikawa Y, Ishii T, Asuwa N, Masuda S. Absence of atherosclerosis evolution in the coronary arterial segment covered by myocardial tissue in cholesterol-fed rabbits. Virchows Arch. 1997; 430(2): 163-71.

78 Chandalia M, Garg A, Vuitch F, Nizzi F. Postmortem findings in congenital generalized lipodystrophy. J.Clin.Endocrinol.Metab 1995; 80(10): 3077-81.

79 Prati F, Arbustini E, Labellarte A, Sommariva L, Pawlowski T, Manzoli A, Pagano A, Motolese M, Boccanelli A. Eccentric atherosclerotic plaques with positive remodelling have a pericardial distribution: a permissive role of epicardial fat? A three-dimensional intravascular ultrasound study of left anterior descending artery lesions. Eur.Heart J. 2003; 24(4): 329-36.

80 Prescott MF, McBride CK, Court M. Development of intimal lesions after leukocyte migration into the vascular wall. Am.J.Pathol. 1989; 135(5): 835-46.

81 Miyata K, Shimokawa H, Kandabashi T, Higo T, Morishige K, Eto Y, Egashira K, Kaibuchi K, Takeshita A. Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. Arterioscler.Thromb.Vasc.Biol. 2000; 20(11): 2351-8.

82 Shimokawa H, Ito A, Fukumoto Y, Kadokami T, Nakaike R, Sakata M, Takayanagi T, Egashira K, Takeshita A. Chronic treatment with interleukin-1 beta induces coronary intimal lesions and vasospastic responses in pigs in vivo. The role of platelet-derived growth factor. J.Clin.Invest 1996; 97(3): 769-76.

83 Henrichot E, Juge-Aubry CE, Pernin A, Pache JC, Velebit V, Dayer JM, Meda P, Chizzolini C, Meier CA. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? Arterioscler.Thromb.Vasc.Biol. 2005; 25(12): 2594-9.

84 Karastergiou K, Evans I, Ogston N, Miheisi N, Nair D, Kaski JC, Jahangiri M, Mohamed-Ali V. Epicardial Adipokines in Obesity and Coronary Artery Disease Induce Atherogenic Changes in Monocytes and Endothelial Cells. Arterioscler.Thromb.Vasc.Biol. 2010.

85 Lohn M, Dubrovska G, Lauterbach B, Luft FC, Gollasch M, Sharma AM. Periadventitial fat releases a vascular relaxing factor. FASEB J. 2002; 16(9): 1057-63.

86 Verlohren S, Dubrovska G, Tsang SY, Essin K, Luft FC, Huang Y, Gollasch M. Visceral periadventitial adipose tissue regulates arterial tone of mesenteric arteries. Hypertension 2004; 44(3): 271-6.

87 Greenstein AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M, Laing I, Yates AP, Pemberton PW, Malik RA, Heagerty AM. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. Circulation 2009; 119(12): 1661-70.

88 Payne GA, Bohlen HG, Dincer UD, Borbouse L, Tune JD. Periadventitial adipose tissue impairs coronary endothelial function via PKC-beta-dependent phosphorylation of nitric oxide synthase. Am.J.Physiol Heart Circ.Physiol 2009; 297(1): 460-465.

89 Payne GA, Borbouse L, Bratz IN, Roell WC, Bohlen HG, Dick GM, Tune JD. Endogenous adipose-derived factors diminish coronary endothelial function via inhibition of nitric oxide synthase. Microcirculation. 2008; 15(5): 417-26.

90 Police SB, Thatcher SE, Charnigo R, Daugherty A, Cassis LA. Obesity Promotes Inflammation in Periaortic Adipose Tissue and Angiotensin II-Induced Abdominal Aortic Aneurysm Formation. Arterioscler.Thromb. Vasc.Biol. 2009.

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Chapter 3

Coronary perivascular adipose tissue characteristics are related to atherosclerotic plaque size and composition A post-mortem study

Atherosclerosis (in revision)

ABSTRACT

Background: Perivascular adipose tissue (pvAT) may influence atherosclerotic plaque formation. We aim to determine the association between the local amount and inflammatory properties of pvAT and the size and composition of atherosclerotic plaque in the left anterior descending artery (LAD).

Methods: Post mortem, a total of 139 cross sections of the LAD were obtained from 16 patients. PvAT quantity was measured within an area of 3 mm around the LAD (pvAT-area (%)). Furthermore, inflammatory properties of pvAT were measured (macrophages /400x field and adipocyte size). From plaque area (mm^2), plaque/media-ratio was calculated and morphologic characteristics were scored (presence of a lipid core and calcification; collagen and smooth muscle cell content; macrophage (<10, 10-50, >50/ 400x field) and lymphocyte infiltration (<10, 10-25, >25/ 400x field).

Results: Plaque/media-ratio increased with increasing pvAT-area (β0.02; 95%CI 0.01-0.03) and pvAT macrophages (β 0.10; 95%CI 0.05-0.16), but not with adipocyte area (β -0.00; 95%CI -0.07-0.06). PvAT-area was related to the presence of a lipid core (OR 1.05; 95%CI 1.03-1.08) and with macrophage and lymphocyte infiltration of atherosclerotic plaque (per increase in category OR 1.05; 95%CI 1.02-1.07 and OR 1.04; 95%CI 1.01-1.07 respectively). PvAT macrophage infiltration was correlated with adventitia and plaque macrophages.

Conclusion: PvAT quantity and macrophage infiltration are highly related to atherosclerotic plaque size and composition in patients with coronary atherosclerosis. These results indicate potential involvement of pvAT in coronary atherosclerotic plaque development, although the causality of the relation has yet to be determined.

INTRODUCTION

Atherosclerotic vascular disease is characterized by focal lesions and areas without atherosclerotic plaque[1]. The focal aspect of atherosclerotic plaque formation cannot be explained by risk factors with a generalized effect on the vasculature such as smoking or hypercholesterolemia, but may be explained by local conditions such as non-laminar hemodynamic flow[2] and local inflammation[3;4].
Obesity is characterized by a state of low grade inflammation, which is involved in the process of atherosclerosis[5;6]. Increased visceral adipose tissue volume induces adipose tissue dysfunction[7] and systemic inflammation[8] as enlarged adipocytes produce a wide range of adipocytokines and chemokines, attracting macrophages to adipose tissue[9]. Crosstalk between adipocytes and macrophages leads to enhanced secretion of inflammatory adipokines and cytokines inducing systemic inflammation that contributes to the development of vascular diseases, insulin resistance and type 2 diabetes[10].
Adipose tissue around arteries, perivascular adipose tissue (pvAT), has been hypothesized to stimulate and induce atherosclerotic plaque formation from outside the artery ('from outside to inside') by induction of a local inflammatory environment[11;12]. Coronary pvAT is part of epicardial adipose tissue and although there is no anatomical border, epicardial adipose tissue is morphologically and functionally different from pvAT[13]. In patients suspected of coronary artery disease, the volume of pvAT on CT is higher near coronary artery segments with atherosclerotic plaque than near coronary artery segments without plaque[14]. Furthermore, infiltrates in the adventitia are related to intima inflammation[15] and there is an over-expression of pro-inflammatory genes in epicardial adipose tissue of patients with coronary artery disease compared to controls[16]. However, to determine whether there is an influence of local adipose tissue on the vascular wall, it is important to delimitate the region of interest and to take both adipose tissue quantity and inflammation into account.
The aim of the present post mortem study was to investigate local properties of pvAT in relation to local atherosclerosis. We investigated the relation between coronary pvAT quantity and inflammation and coronary plaque size and composition in cross sections of the left anterior descending coronary artery (LAD).

METHODS

A total of 16 patients with coronary atherosclerosis were included in the study between April 2009 and February 2011. Patients were referred for routine autopsy to the department of Pathology of the University Medical Centre in Utrecht the Netherlands. Eligible patients had a vascular cause of death or were > 40 years of age. Moreover, they did not fulfil any of the exclusion criteria (history of PTCA, CABG, kidney failure, recent sepsis, known reduction of body weight (>5%) in the past 2 years, malignancy in the past 2 years, thyroid disease or oral use of steroid hormones). In the majority of patients (75%) death was caused by acute myocardial infarction. In the other cases death was caused by aspiration pneumonia following ischemic stroke, by haemorrhagic stroke, ruptured aortic aneurysm and a work-related accident. The study met the criteria of the code of proper use human tissue that is used in the Netherlands for the use of human tissue.

Processing of coronary artery specimens

Bodies were stored at 4°C and autopsy was performed by the attending pathologist. The LAD was prepared from the heart with the surrounding adipose tissue and underlying myocardium taken along in a radius of 1-2 centimetres (figure 1a). The coronary arteries were fixed in formaldehyde 4% for one day. Subsequently the material was decalcified in ethylenediaminetetra-acetic acid (EDTA) solution during 2 to 5 days depending on the extent of calcification.

Coronary arteries were cross sectioned, starting at the origin of the LAD (bifurcation of LAD and left circumflex coronary artery), at 5 mm intervals. Subsequently cross sections where embedded in paraffin. Only cross sections within 6 cm from the origin of the LAD (n=143) were analysed, as LAD-specimens of more than 6 cm from the origin of the LAD were only available in 4 cases. Cross sections with an intra-myocardial localization of the LAD, referred to as myocardial bridge, were excluded because of virtual absence of adipose tissue. Mean distance to the origin of the LAD was 3 cm. Proximal cross sections were defined as cross sections within the first 3 cm and distal cross sections as within 3 to 6 cm from the origin of the LAD.

Histology and immuno-histochemical staining

Thin sections of 4 μm were processed for histological staining with haematoxylin & eosin (H&E), Picro-Sirius red, and elastin von Gieson. In addition immuno-histochemistry stainings were performed to quantify macrophages (CD68), lymphocytes (CD3 and CD20) and smooth muscle cells (α-smooth muscle actin).

For measurement of perivascular adipose tissue and atherosclerotic plaque areas, elastin von Gieson histology specimens were digitalized and analysed using Image Pro plus software (Mediacybernetics).

Perivascular adipose tissue quantity and characteristics

To determine the quantitative exposure of the coronary arteries to adipose tissue, pvAT-thickness (mm) was measured at the level of the lateral vessel wall, from the myocardium to the pericardium. PvAT-area (%) was defined as the percentage of adipose tissue within the area around the vessel. The area was delimitated by a circle with the radius of the vessel including the adventitia and an additional 3 mm (supplemental figure 1a). The intra-class correlation (ICC) of the pvAT-area (%) measurement with a 3 mm circle and pvAT-area (%) with a 5 mm circle around the vessel was 0.85 ($p<0.001$).

Macrophage infiltration and adipocyte size were measured in pvAT in a region within 3 mm of the coronary artery. Macrophage infiltration was scored by determining the mean number of CD68-positive cells in 10 high power fields (HPF; magnification 400x) in all quadrants of the coronary artery (supplemental figure 1c). The adipocyte size was determined, by calculating the mean adipocyte area of 100 adipocytes in 100 randomly selected fields at 200x magnification (supplemental figure 1b)[17]. The ICC's for pvAT macrophages and adipocyte size were 0.76 ($p<0.001$) and 0.90 ($p<0.001$) respectively.

Atherosclerotic plaque quantity and characteristics

Atherosclerotic plaque was defined as the area (mm^2) between the lumen and the internal elastic lamina (IEL) and the media was defined as the area (mm^2) between the IEL and external elastic lamina (EEL)[4]. To account for arterial tapering the percentage of vessel area consisting of plaque

was calculated (lumen stenosis). To account for arterial remodelling the ratio of the plaque area to the media was calculated (plaque/media-ratio).
The morphologic characteristics of the plaque were scored by the first author and each case was subsequently reviewed by the second author (supplemental figure 2). The final score was given with mutual agreement. Presence of a lipid rich core was scored on H&E- and Picro-Sirius red stainings and presence of plaque calcification on H&E and on haematoxylin counterstaining of immunohistochemistry slides. Collagen and smooth muscle cell content of atherosclerotic plaques was determined semi-quantitatively on Picro-Sirius red and α-smooth muscle actin stainings respectively. High collagen or smooth muscle cell plaque content was defined as presence of these determinants in >50% of the plaque area and low content as <50%. Macrophages were examined on CD68 stains and for lymphocytes the categorization was based on CD3 and CD20 stains. Macrophages and lymphocytes in plaque and adventitia were scored in 4 categories. Plaque macrophages in the categories: no macrophages, few scattered, groups of 10-50, and >50.
Plaque lymphocytes in the categories: no lymphocytes, groups of <10, 10-25, and >25. Adventitia macrophages in the categories: no macrophages, <10, 10-25, and >25 per HPF (400x). Adventitial lymphocytes in the categories: no lymphocytes, scattered, aggregates of >25, and >100 in at least 1 quadrant. In the analyses the first 2 categories were taken together, because cases in these 2 categories did not differ in adipose tissue measures.

Data analyses

If normally distributed, continuous variables are displayed as mean with standard deviation. To explore the confounding effect of distance to the origin of the LAD on the relation between pvAT and plaque characteristics, differences between cross sections close to the origin of the artery and more distal sections were tested. Normally distributed variables were tested with independent samples t-tests. Variables with skewed distributions are presented as median with interquartile range (IQR) and Kruskal-Wallis analyses were used to test for differences. Numbers with percentage of ordinal variables are displayed and differences were tested with Chi-square.
The relations between pvAT characteristics and continuous, dichotomous and ordinal plaque characteristics were evaluated with regression analyses. In order to take clustering of characteristics within subjects into account, general estimated equations analysis with a compound symmetry structure was used in all regression analyses. Models were linear for relations between continuous measures of adipose tissue and plaque size. Although variables were skewed, residuals in linear regression analyses were considered normally distributed and therefore no transformations were made to the dependent and independent variables. Binary logistic models were used for dichotomous plaque characteristics and ordinal logistic models for categorical plaque characteristics. Relations were explored in a univariable model (model I) and in models with distance to the origin of the LAD (model II) as a covariable. Analyses with pvAT-area were adjusted for pvAT-macrophages and adipocyte size in additional analyses. General estimated equations analyses with pvAT macrophages were repeated excluding LAD cross sections with intra-plaque haemorrhage or thrombus. Further sensitivity analyses were performed by analysing data from cases with AMI only (n=12; 105 cross sections).

RESULTS

Patient characteristics

Characteristics of patients are outlined in table 1. Mean age was 63±13 years and 10 out of 16 subjects were male (63%). Per patient, a mean of 9±2 cross sections from 0.5 to 6.0 cm from the origin of the LAD were available. Four cross sections from 2 subjects were not included in the analyses, because of presence of a myocardial bridge, leaving 139 cross sections available for analyses. In 6 out of 12 subjects with AMI, the culprit lesion was located in the LAD. In the other 6 cases, the culprit lesion was located in de circumflex or right coronary artery. A number of 9 cross sections of 6 subjects showed intra plaque haemorrhage and 3 cross sections of 3 subjects showed thrombus in the LAD.

Table 1 | Characteristics of autopsy cases (n=16)

Age (years)	63±12
Males, n	10 (63%)
Ethnicity white, n	15 (94%)
Weight (kg)	85±16
Cause of death AMI, n	12 (75%)
Type 2 diabetes, n	3 (19%)
Smoking, n	8 (67%)
Hypertension, n	7 (44%)
Statin use, n	2 (13%)
Occlusion LAD, n	6 (38%)

AMI; acute myocardial infarction; LAD: left anterior descending coronary artery

Perivascular adipose tissue and atherosclerosis in relation to distance to origin of the LAD

PvAT-thickness was 9.5±2.6 mm in proximal and 6.9±2.4 mm in distal sections (table 2). Also pvAT-area (%), a measure not affected by tapering of the coronary artery, was increased in proximal cross sections (83; IQR 74-96 and 80; IQR 69-89 respectively). Atherosclerotic plaque was more pronounced in proximal than in distal cross sections with a plaque/media-ratio 2.2 (IQR 1.2-3.6) versus 1.4 (IQR 0.9-2.6) and lesions were more advanced with a lipid core of 33% versus 13%.

Perivascular adipose tissue in relation to plaque size

There was a relation between pvAT quantity expressed as pvAT-area and plaque area (mm^2) (β pvAT-area (%) 0.07; 95%CI 0.04-0.10), also after adjustment for distance to the origin of the LAD (table 3a). The relation was still present with plaque/media-ratio, a measure of plaque size taking outward remodelling into account (β pvAT-area (%) 0.02; 95%CI 0.01-0.03). Inflammation of pvAT expressed as pvAT-macrophages (n/HPF) was related to plaque/media-ratio (β 0.10; 95%CI 0.05-0.16), but adipocyte size was not (β -0.00; 95%CI -0.07-0.06).

Perivascular adipose tissue in relation to plaque composition

An increase in pvAT-area (%) was associated with these morphologic plaque characteristics (table 3b), although the relation between pvAT-area (%) and collagen content was not (OR high versus low content 0.92; 95%CI 0.83-1.01). Increasing pvAT-area (%) was associated with an increase in plaque macrophages (per category increase OR 1.05; 95%CI 1.02-1.07) and plaque lymphocytes (OR 1.04; 95%CI 1.01-1.07) (figure 3).

With an increase of pvAT-macrophages (n/HPF), plaques more often showed a lipid core, calcification and a low collagen content (OR high versus low collagen content 0.84; 95%CI 0.78-0.90).

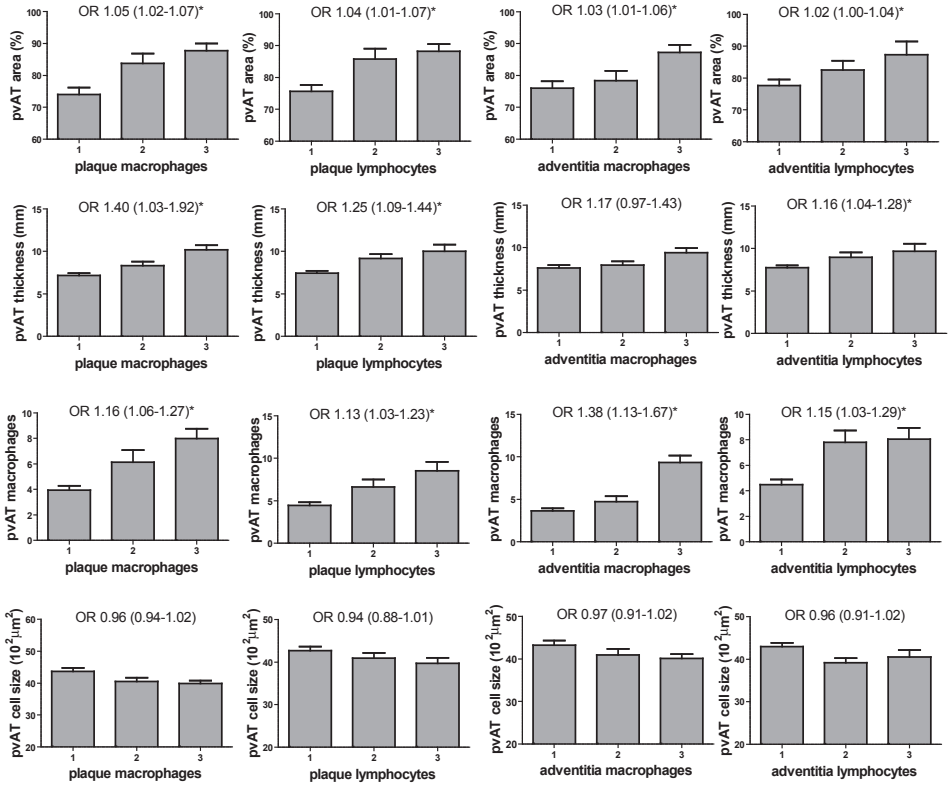


Figure 1 | Perivascular adipose tissue (pvAT) characteristics in relation to atherosclerotic plaque inflammation

*Bars indicate means with SEM. *P<0.05 Data are analysed with generalized estimating models (GEE) taking clustering within subjects into account. OR's (95%CI) indicate the chance per unit of the pvAT characteristic of being in a higher category of plaque inflammation.*

Furthermore, pvAT-macrophages (n/HPF) were associated with macrophage infiltration in plaque and in the adventitia (per category increase OR 1.16; 1.06-1.27 and OR 1.38; 95%CI 1.13-1.67 respectively) (figure 1). PvAT -macrophages (n/HPF) were also associated with plaque and adventitia lymphocytes (per category increase OR 1.13; 95%CI 1.03-1.23 and OR 1.15; 95%CI 1.03-1.29 respectively). There was no relation between adipocyte cell size and plaque composition. Overall, adjustment for distance to the origin of the LAD (model II) did not substantially change the point estimates for the relations between pvAT and plaque composition.

Sensitivity analyses were performed by analysing data from the 12 cases with AMI only. In AMI cases relations between pvAT and atherosclerotic plaque were comparable to the whole group. Analyses excluding the 12 cross sections with intra plaque haemorrhage or thrombus did not change the relation between pvAT-macrophages (n/HPF) and plaque size and composition. Moreover, repeating the analyses with pvAT-area as independent variable, using pvAT-macrophages and adipocyte size as covariates, did not change the point estimates of the relations.

Table 2 | Perivascular adipose tissue (pvAT) and plaque characteristics according to distance to origin of the LAD.

	Proximal LAD (0-3cm)	Distal LAD (3-6cm)	p-value
	n=76	n=63	
pvAT-area (%)	83 (74-96)	80 (69-89)	0.03
pvAT macrophages (n/HPF)	4.7 (3.0-7.2)	3.5 (2.5-6.0)	0.07
Adipocyte cell size (μm^2)	4051±793	4342±727	0.03
EEL (mm^2)	9.8±4.5	5.0±2.4	<0.001
Lumen (mm^2)	2.5 (1.3-3.7)	1.2 (0.5-1.8)	<0.001
Plaque area (mm^2)	4.6 (2.2-6.7)	1.8 (0.8-3.8)	<0.001
Lumen stenosis (%)	63±19	59±24	0.43
Plaque/media-ratio	2.2 (1.2-3.6)	1.4 (0.9-2.6)	0.01
Lipid core present (n)	25 (33%)	8 (13%)	0.01
Calcification present (n)	29 (38%)	13 (21%)	0.03
Collagen (>50%) (n)	70 (92%)	60 (95%)	0.46
Smooth muscle cells (>50%) (n)	44 (58%)	46 (73%)	0.06
Plaque macrophages (>10/ HPF) (n)	48 (63%)	24 (38%)	0.01
Plaque lymphocytes (>10/ HPF) (n)	36 (47%)	18 (29%)	0.03
Adventitia macrophages (>10/ HPF) (n)	45 (59%)	26 (41%)	0.04
Adventitia lymphocytes (presence of aggregates of >25) (n)	32 (42%)	15 (24%)	0.02

Mean±SD of variables with normal distribution are presented and median (interquartile range) of variables with a skewed distribution. Categorical variables are presented as number with percentage. HPF: high power field; EEL: external elastic lamina

Table 3a | Perivascular adipose tissue (pvAT) in relation to atherosclerotic plaque size

	Model	Plaque area (mm^2) β (95%CI)	Lumen stenosis (%) β (95%CI)	Plaque/media-ratio β (95%CI)
pvAT-area (%)	I	0.07 (0.04-0.10)*	0.21 (0.05-0.38)*	0.02 (0.01-0.03)*
	II	0.04 (0.01-0.07)*	0.18 (-0.05-0.42)*	0.02 (0.00-0.03)*
pvAT-thickness (mm)	I	0.54 (0.28-0.80)*	0.84 (-0.42-2.10)	0.13 (0.02-0.25)*
	II	0.38 (0.10-0.67)*	0.65 (-1.29-2.58)	0.15 (0.07-0.22)*
pvAT macrophages (n/HPF)	I	0.14 (-0.02-0.29)	0.99 (0.39-1.58)*	0.10 (0.05-0.16)*
	II	0.09 (-0.04-0.22)	1.06 (0.09-2.02)*	0.13 (0.03-0.22)*
Adipocyte cell size (per 100 μm^2)	I	-0.08 (-0.14- -0.01)*	0.13 (-0.54-0.80)	-0.00 (-0.07-0.06)
	II	-0.06 (-0.16-0.04)	0.11 (-0.64-0.86)	0.01 (-0.05-0.07)

*P<0.05; model I: unadjusted; model II: adjusted for distance to the origin of the LAD
pvAT-area: % of pvAT within the perivascular area: a circle of 3 mm drawn around the vessel; pvAT macrophages: mean number of CD68+ cells in 10 high power fields (HPF); adipocyte cell size: mean adipocyte area of 100 pvAT adipocytes in 100 randomly selected fields
Data are analysed with generalized estimating models (GEE) taking clustering within subjects into account

Table 3b | Perivascular adipose tissue (pvAT) in relation to atherosclerotic plaque composition

	Model	Lipid core OR (95%CI)	Calcification OR (95%CI)	Collagen OR (95%CI)	Smooth muscle cells OR (95%CI)
pvAT-area (%)	I	1.05 (1.03-1.08)*	1.04 (1.02-1.06)*	0.92 (0.83-1.01)	0.96 (0.94-0.98)*
	II	1.04 (1.03-1.06)*	1.03 (1.01-1.04)*	0.92 (0.82-1.03)	0.97 (0.96-0.99)*
pvAT-thickness (mm)	I	1.29 (1.10-1.51)*	1.30 (1.05-1.60)*	0.83 (0.67-1.04)	0.75 (0.63-0.88)*
	II	1.22 (1.01-1.46)*	1.22 (1.00-1.48)*	0.92 (0.82-1.03)	0.90 (0.71-1.13)
pvAT macrophages (n/HPF)	I	1.22 (1.09-1.37)*	1.13 (1.02-1.24)*	0.84 (0.78-0.90)*	0.91 (0.82-1.02)
	II	1.20 (1.07-1.37)*	1.12 (1.02-1.23)*	0.85 (0.78-0.92)*	0.91 (0.82-1.00)
Adipocyte cell size (per 100 μm^2)	I	0.97 (0.91-1.02)	0.94 (0.88-1.00)	1.05 (0.93-1.19)	1.06 (1.00-1.12)
	II	1.00 (0.95-1.05)	0.99 (0.94-1.04)	1.02 (0.93-1.13)	1.00 (0.93-1.06)

*P<0.05; model I: unadjusted; model II: adjusted for distance to the origin of the LAD
pvAT-area: % of pvAT within the perivascular area: a circle of 3 mm drawn around the vessel; pvAT macrophages: mean number of CD68+ cells in 10 high power fields (HPF); adipocyte cell size: mean adipocyte area of 100 pvAT adipocytes in 100 randomly selected fields
Data are analysed with generalized estimating models (GEE) taking clustering within subjects into account

DISCUSSION

In this post mortem study pvAT quantity, measured as pvAT-area (%), was related to atherosclerotic plaque size and composition in the LAD. In addition pvAT-macrophages, but not adipocyte cell size of pvAT, was related to atherosclerotic plaque size and composition. In most studies the total amount of epicardial adipose tissue, instead of the adipose tissue directly around the vessel is investigated in relation to coronary atherosclerosis, because of difficulties in delimitation of pvAT. In the present study adipose tissue directly around the coronary artery was studied. Measuring this pvAT is of most interest because of the close anatomical relation between this adipose tissue and the coronary arteries.

We observed an association between the amount of pvAT and atherosclerotic plaque size in the adjacent vascular wall, which is in accordance with previous results. In two studies with patients suspected of coronary artery disease or acute coronary syndrome, local atherosclerosis was highly related to the local volume of pvAT as measured with CT[14;18]. PvAT was 27 cm^3 over the proximal 4 centimetres in one study[14] and 0.5 cm^3 per coronary artery segment in the other study[18]. The wide range in pvAT volumes that was observed may be due to differences in definitions of pvAT.

Quantity of pvAT and atherosclerotic plaque decreases from proximal to distal portions of the coronary artery[1;11]. This may confound the relationship between the two variables, although one can argue that a relation of pvAT and plaques with distance to the origin of the coronary artery supports the theory of a causal relation between local adipose tissue and atherosclerosis. To overcome this confounding effect, we adjusted for distance to the origin of the LAD. With this adjustment, the relation between adipose tissue and plaque quantity was not affected. In addition we used measures of pvAT and plaque size that take tapering and remodelling of the coronary artery into account[4;19].

To the best of our knowledge this is the first study where pvAT characteristics are investigated in relation to plaque composition. Plaque characteristics such as presence of a lipid core, plaque macrophages and a decrease in smooth muscle cells are markers of plaque instability[20]. The presence of a lipid core and a high macrophage content are more common in patients with ruptured plaque

than in patients with thin cap fibro-atheroma[20]. Although referred to as markers of plaque instability, low collagen and plaque calcification content in atherosclerotic plaques are less established as risk factors of plaque rupture[20;21]. In the present study, an increase in pvAT was related to the presence of a lipid core and with the presence of calcification in plaque. In congruence, the total volume of the epicardial adipose tissue depot and the thickness of pvAT measured on CT images is higher in patients with atherosclerotic plaques compared to patients without plaques in the coronary arteries[22;23]. Patients with mixed plaques, consistent with a fibro-atheromatous lesions, have higher epicardial adipose tissue volume than patients with calcified plaques or no plaques[22].

Furthermore, pvAT is higher around coronary arteries with mixed plaques compared to other plaque types[14]. In support of the relation between pvAT characteristics and calcified plaques in the current study, there is an association between epicardial adipose tissue volume and calcium score[22;23] and between expansion of epicardial adipose tissue and an increase in the number of calcified plaques[24]. In the present study macrophage infiltration in pvAT was associated with plaque size and plaque composition. These results are in concordance with findings in 4 patients[25]. In pvAT near the atherosclerotic aorta, macrophages were more abundant than in pvAT near non-atherosclerotic peripheral arteries of the same patient. No information on plaque composition was mentioned in this study. The association between macrophages and atherosclerotic plaque also holds true for epicardial adipose tissue. Medium conditioned by human epicardial adipose tissue is able to induce migration of monocytes in vitro[26]. Moreover, in a recent study biopsies of epicardial fat were taken during cardiac surgery[27]. In patients with coronary atherosclerosis, the pro-inflammatory M1 macrophages in epicardial adipose tissue were more prominent than in patients without coronary atherosclerosis.

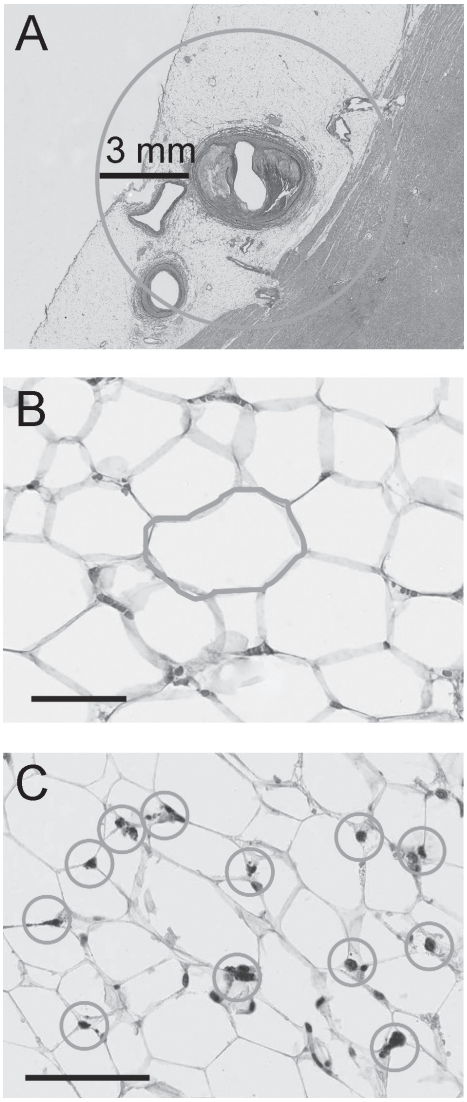
Furthermore, inflammatory cell invasion in the adventitia is associated with the extent of local atherosclerosis in patients with severe CAD[15]. Moreover, adventitial lymphocytic aggregates and an increase in adventitial macrophage content, are well correlated with markers of plaque instability and plaque size[15;28]. Therefore, inflammation of surrounding tissues may be a continuous process. Not only is adventitial inflammation associated with atherosclerosis in other studies[15;28], the inflammation in pvAT was correlated with adventitial inflammation in the present study.

In contrast with other studies, we did not observe a relation between adipocyte size and inflammation. Adipocyte enlargement is thought to result in a pro-inflammatory state in subcutaneous and visceral adipose tissue[29]. Adipocyte size in pvAT and plaque characteristics were not related in the present study. However, perivascular adipocytes differ from adipocytes in subcutaneous adipose tissue and therefore this may not apply for all adipose tissue depots. Perivascular adipocytes are smaller and more irregular in shape than subcutaneous adipocytes[13]. In epicardial adipose tissue the expression of MCP-1 decreases with adipocyte enlargement[30], indicating that small adipocytes have increased pro-inflammatory properties compared to larger adipocytes. This is in congruence with the finding in the present study that adipocyte size is negatively related to plaque area, although there is no association with other plaque characteristics.

In this study we have evaluated different pvAT and plaque characteristics. The observed results all point in the same direction, thereby consistently supporting the hypothesis that pvAT may affect atherosclerosis. However, this is a cross sectional study and therefore it is not possible to make inferences about causality. It may well be possible that the relation is the other way around. It can be speculated that signals from the vessel wall influence the surrounding adipose tissue. The study population of patients with coronary artery disease showed a distribution of atherosclerotic burden between and within subjects. Due to sample size it was not possible to analyse characteristics between

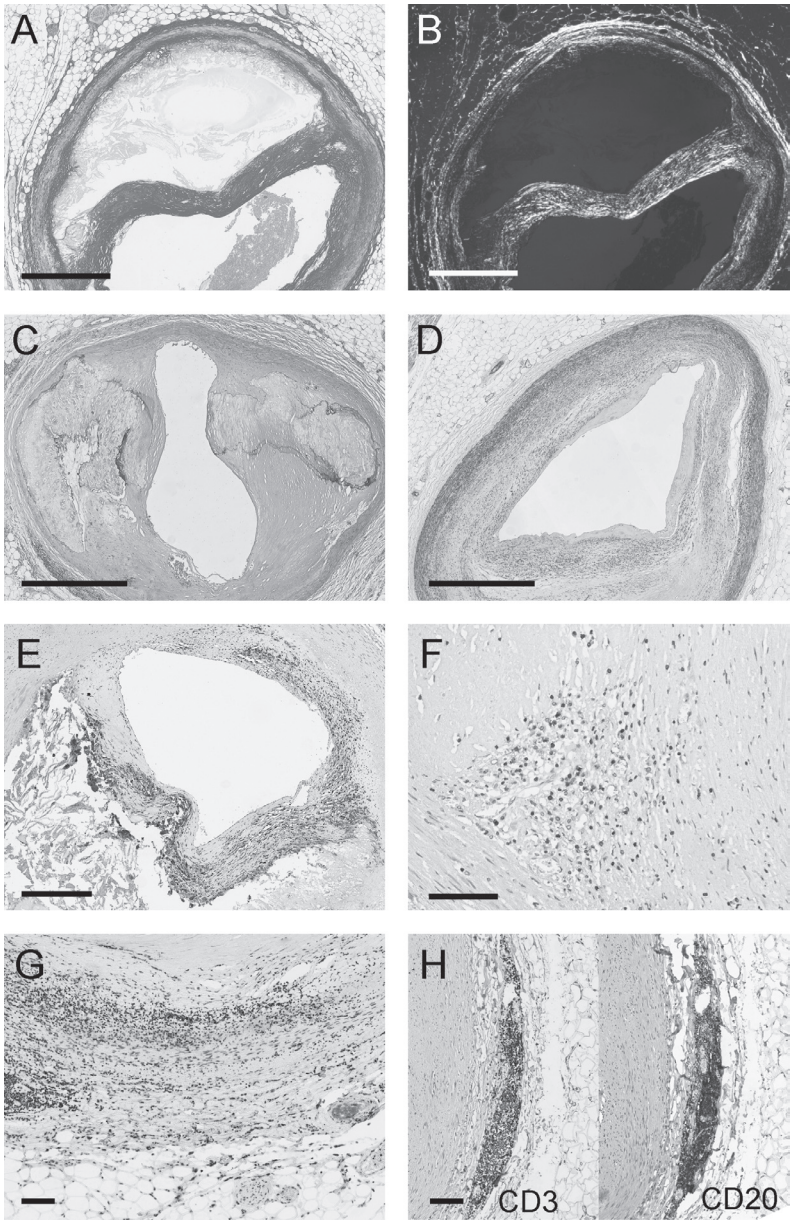
subjects. Generalized estimating equations analysis was applied to overcome variance caused by clustering of pvAT and plaque characteristics. Furthermore, it was not possible to analyse plaque morphology in a quantitative fashion. The semi-quantitative measures of plaque characteristics that were used, have been shown to be well reproducible in other studies[31]. Finally we did not have measures of obesity available in all patients. It would have been interesting to know whether the relation between pvAT and atherosclerotic plaque is partly driven by obesity.

In conclusion, pvAT quantity and macrophage infiltration are related to quantitative, morphologic and inflammatory plaque properties in patients with coronary artery disease. These results support the concept that pvAT may play a role in the development of coronary atherosclerosis, although the causality of the relation has yet to be determined.



Supplemental figure 1 | Methods
A: Measurement of pvAT(%): the percentage of adipose tissue area within the area of the circle (radius of 3 mm outside of the adventitia); B, measurement of adipocyte area Bar = 50 μm; C, pvAT macrophages Bar = 100 μm.

See page 146 for a full color representation of this figure



Supplemental figure 2 | Histology of plaque and adventitia.
A, Picrosirius red staining showing a lipid core (>40% of plaque area: category 3); collagen content (<50% of plaque area). Bar = 1mm. B, Picrosirius red staining with polarized light. Bar = 1mm. C, H&E-staining of a plaque with calcification (10-40% of plaque area: category 2). Bar = 1mm. D, αSMA staining with smooth muscle cells (> 50% of plaque area). E, CD68 staining with groups of >50 macrophages in the plaque (category 3). Bar = 500 μm. F, CD3 staining showing a group of >25 lymphocytes in the plaque shoulder (category 3). Bar = 100 μm. G, CD68 staining showing >25 adventitia macrophages (category 3). The upper side of photograph: plaque, at the bottom: adventitia; Bar = 100 μm. H, CD3 and CD20 stainings of the adventitia showing an aggregate of >100 lymphocytes in the adventitia (category 3) consisting of T- and B-cells. Bar = 100 μm.

See page 147 for a full color representation of this figure

REFERENCE LIST

1 Cheruvu PK, Finn AV, Gardner C, Caplan J, Goldstein J, Stone GW et al. Frequency and distribution of thin-cap fibroatheroma and ruptured plaques in human coronary arteries: a pathologic study. J Am Coll Cardiol 2007; 50(10):940-949.

2 Shinjo SK, Prates NE, Oba SM, Sampaio LO, Nader HB. Distribution and composition of glycosaminoglycans in the left human coronary arterial branches under myocardial bridge. Atherosclerosis 1999; 143(2):363-368.

3 Pasterkamp G, Schoneveld AH, van der Wal AC, Hijnen DJ, van Wolveren WJ, Plomp S et al. Inflammation of the atherosclerotic cap and shoulder of the plaque is a common and locally observed feature in unruptured plaques of femoral and coronary arteries. Arterioscler Thromb Vasc Biol 1999; 19(1):54-58.

4 Vink A, Schoneveld AH, Richard W, de Kleijn DP, Falk E, Borst C et al. Plaque burden, arterial remodeling and plaque vulnerability: determined by systemic factors? J Am Coll Cardiol 2001; 38(3):718-723.

5 Libby P. Inflammation in atherosclerosis. Nature 2002; 420(6917):868-874.

6 Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999; 340(2):115-126.

7 Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG et al. Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham Heart Study. Circulation 2007; 116(11):1234-1241.

8 Faber DR, van der Graaf Y, Westerink J, Visseren FL. Increased visceral adipose tissue mass is associated with increased C-reactive protein in patients with manifest vascular diseases. Atherosclerosis 2010; 212(1):274-280.

9 Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 2008; 29(24):2959-2971.

10 Suganami T, Tanimoto-Koyama K, Nishida J, Itoh M, Yuan X, Mizuarai S et al. Role of the Toll-like receptor 4/NF-kappaB pathway in saturated fatty acid-induced inflammatory changes in the interaction between adipocytes and macrophages. Arterioscler Thromb Vasc Biol 2007; 27(1):84-91.

11 Sacks HS, Fain JN. Human epicardial adipose tissue: a review. Am Heart J 2007; 153(6):907-917.

12 Verhagen SN, Visseren FL. Perivascular adipose tissue as a cause of atherosclerosis. Atherosclerosis 2011; 214(1):3-10.

13 Chatterjee TK, Stoll LL, Denning GM, Harrelson A, Blomkalns AL, Idelman G et al. Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. Circ Res 2009; 104(4):541-549.

14 Maurovich-Horvat P, Kallianos K, Engel LC, Szymonifka J, Fox CS, Hoffmann U et al. Influence of pericoronary adipose tissue on local coronary atherosclerosis as assessed by a novel MDCT volumetric method. Atherosclerosis 2011.

15 Tavora F, Kutys R, Li L, Ripple M, Fowler D, Burke A. Adventitial lymphocytic inflammation in human coronary arteries with intimal atherosclerosis. Cardiovasc Pathol 2010; 19(3):e61-e68.

16 Sacks HS, Fain JN, Cheema P, Bahouth SW, Garrett E, Wolf RY et al. Depot-Specific Overexpression of Proinflammatory, Redox, Endothelial Cell, and Angiogenic Genes in Epicardial Fat Adjacent to Severe Stable Coronary Atherosclerosis. Metab Syndr Relat Disord 2011.

17 Faber DR, Moll FL, Vink A, van der Waal C, Kalkhoven E, Schipper HS et al. Adipose tissue quantity and composition contribute to adipokine concentrations in the subclavian vein and the inferior mesenteric vein. Int J Obes (Lond) 2011.

18 Mahabadi AA, Reinsch N, Lehmann N, Altenbernd J, Kalsch H, Seibel RM et al. Association of pericoronary fat volume with atherosclerotic plaque burden in the underlying coronary artery: a segment analysis. Atherosclerosis 2010; 211(1):195-199.

19 Schwartz RS, Topol EJ, Serruys PW, Sangiorgi G, Holmes DR, Jr. Artery size, neointima, and remodeling: time for some standards. J Am Coll Cardiol 1998; 32(7):2087-2094.

20 Kolodgie FD, Burke AP, Farb A, Gold HK, Yuan J, Narula J et al. The thin-cap fibroatheroma: a type of vulnerable plaque: the major precursor lesion to acute coronary syndromes. Curr Opin Cardiol 2001; 16(5):285-292.

21 Davies MJ, Richardson PD, Woolf N, Katz DR, Mann J. Risk of thrombosis in human atherosclerotic plaques: role of extracellular lipid, macrophage, and smooth muscle cell content. Br Heart J 1993; 69(5):377-381.

22 Alexopoulos N, McLean DS, Janik M, Arepalli CD, Stillman AE, Raggi P. Epicardial adipose tissue and coronary artery plaque characteristics. Atherosclerosis 2009.

23 Gorter PM, de Vos AM, van der Graaf Y, Stella PR, Doevendans PA, Meijs MF et al. Relation of epicardial and pericoronary fat to coronary atherosclerosis and coronary artery calcium in patients undergoing coronary angiography. Am J Cardiol 2008; 102(4):380-385.

24 Nakanishi R, Rajani R, Cheng VY, Gransar H, Nakazato R, Shmilovich H et al. Increase in epicardial fat volume is associated with greater coronary artery calcification progression in subjects at intermediate risk by coronary calcium score: a serial study using non-contrast cardiac CT. Atherosclerosis 2011; 218(2):363-368.

25 Henrichot E, Juge-Aubry CE, Pernin A, Pache JC, Velebit V, Dayer JM et al. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? Arterioscler Thromb Vasc Biol 2005; 25(12):2594-2599.

26 Karastergiou K, Evans I, Ogston N, Miheisi N, Nair D, Kaski JC et al. Epicardial adipokines in obesity and coronary artery disease induce atherogenic changes in monocytes and endothelial cells. Arterioscler Thromb Vasc Biol 2010; 30(7):1340-1346.

27 Hirata Y, Tabata M, Kurobe H, Motoki T, Akaike M, Nishio C et al. Coronary atherosclerosis is associated with macrophage polarization in epicardial adipose tissue. J Am Coll Cardiol 2011; 58(3):248-255.

28 Moreno PR, Purushothaman KR, Fuster V, O'Connor WN. Intimomedial interface damage and adventitial inflammation is increased beneath disrupted atherosclerosis in the aorta: implications for plaque vulnerability. Circulation 2002; 105(21):2504-2511.

29 Winkler G, Kiss S, Keszthelyi L, Sapi Z, Ory I, Salamon F et al. Expression of tumor necrosis factor (TNF)-alpha protein in the subcutaneous and visceral adipose tissue in correlation with adipocyte cell volume, serum TNF-alpha, soluble serum TNF-receptor-2 concentrations and C-peptide level. Eur J Endocrinol 2003; 149(2):129-135.

30 Eiras S, Teijeira-Fernandez E, Salgado-Somoza A, Couso E, Garcia-Caballero T, Sierra J et al. Relationship between epicardial adipose tissue adipocyte size and MCP-1 expression. Cytokine 2010; 51(2):207-212.

31 Hellings WE, Pasterkamp G, Vollebregt A, Seldenrijk CA, de Vries JP, Velema E et al. Intraobserver and interobserver variability and spatial differences in histologic examination of carotid endarterectomy specimens. J Vasc Surg 2007; 46(6):1147-1154.

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Chapter 4

Secretion of adipocytokines by perivascular adipose tissue near stenotic and non-stenotic coronary artery segments in patients undergoing CABG

Submitted

ABSTRACT

Objective: Perivascular adipose tissue (pvAT) may induce a local pro-inflammatory environment, possibly contributing to coronary atherosclerosis. We investigated whether there is a difference in adipocytokine production by pvAT near stenotic and non-stenotic coronary artery segments in patients with coronary artery disease (CAD).
Design: cross sectional comparison of 2 samples within patients
Patients: patients undergoing CABG with or without valve replacement (n=38).
Interventions: pvAT near stenotic and near non-stenotic coronary segments was harvested and incubated *ex vivo* for 24 hours.
Outcome measures: concentrations of 23 adipocytokines were measured in the supernatants with a Multiplex-assay. The number of macrophages (CD68, CD11c, CD206) and lymphocytes (CD45) in pvAT was determined. Differences between stenosis and control pvAT were tested with Wilcoxon signed rank test corrected for multiple comparisons.
Results: Production of IL-5, IL-1α, IL-17, IL-18 and IL-23 was higher in control than stenosis pvAT samples ($p<0.0021$). Macrophages were more abundant in stenosis than in control pvAT (median n/400x field: 2.3 IQR: 0.3-4.5 versus 1.2 IQR: 0.1-2.5). There was a predominance of M2 macrophages in both stenosis and control pvAT (median n/400x field: macrophages stenosis: M1: 0.0; M2: 1.0 $p=0.004$; control: M1: 0.0; M2: 0.6 $p=0.013$). The relation between adipocytokine production and macrophage infiltration was not different in stenosis and control pvAT.
Conclusion: In patients with CAD, multiple adipocytokines were secreted at higher levels by pvAT near non-stenotic than near stenotic coronary artery segments. Furthermore, pvAT macrophages are associated with stenosis of the adjacent vessel. M2 macrophages were more abundant than M1 macrophages in pvAT.

INTRODUCTION

Rather than being solely a storage depot for triglycerides, adipose tissue is able to secrete pro- and anti-inflammatory cytokines and adipokines[1;2]. Adipose tissue consists of adipocytes, macrophages and a stromal fraction, all contributing to the secretory function[3;4]. Large quantities of adiponectin, leptin and other adipcytokines secreted by adipocytes are released in the systemic circulation[5]. Increased plasma levels of these pro-inflammatory adipocytokines are thought to contribute to processes leading to atherosclerotic disease[6].

In addition to these systemic effects, with abdominal adipose tissue as the major source of systemic concentrations of adipocytokines, adipose tissue around arteries may influence the atherosclerotic process by direct paracrine signalling. Adipose tissue around arteries, often referred to as perivascular adipose tissue (pvAT) is situated close to the adventitia. PvAT may induce a local pro-inflammatory environment in the vascular wall by local secretion of adipocytokines[7;8]. Adipocytokines secreted by pvAT are able to diffuse into the vascular wall and attract macrophages as shown by development of intimal lesions after perivascular application of pro-inflammatory molecules in pigs[9;10]. Furthermore, pvAT is able to induce atherosclerosis in apo-E deficient mice[11].

In epicardial adipose tissue of patients with coronary artery disease (CAD), secretion of interleukin (IL)-6 and leptin is higher and adiponectin lower than of patients without CAD[12]. In addition, the ratio of pro-inflammatory M1 and anti-inflammatory M2 macrophages is more pronounced in epicardial adipose tissue of CAD patients compared to patients without CAD[13]. In these studies factors such as age, gender and body-mass index (BMI) may confound the relation between adipocytokine secretion of epicardial adipose tissue and the presence of CAD. A way to overcome these confounding factors is to investigate differences within the same patient. In 11 patients with CAD, secretion of adiponectin by pvAT near a stented artery was not significantly lower than by pvAT near a control coronary artery segment[14]. Aim of the present study was to evaluate differences in histologic markers of inflammation and differences in the *ex vivo* production of adipocytokine secretion from pvAT in the proximity of coronary atherosclerosis compared to pvAT near coronary artery segments without atherosclerosis in patients undergoing CABG, thus evaluating the potential contribution of pvAT to coronary atherosclerosis by direct paracrine signalling.

METHODS

Patients

From December 2009 to July 2011, 38 patients scheduled for elective coronary artery bypass graft (CABG) surgery with or without valve replacement at the University Medical Centre Utrecht were included in the study. Inclusion criteria thus were presence of ≥50% stenosis of the right coronary artery, left anterior descending artery (LAD), diagonal branch of the LAD or marginal obtusis branches of the circumflex artery. Exclusion criteria were thyroid disease, a history of malignancy in the past 2 years, renal failure (MDRD <30 ml/min/1.73m^2), a history of cardiothoracic surgery and use of thiazolidinediones or immune suppressive medication. In 4 patients pvAT biopsies were not incubated due to absence of qualified laboratory personnel at the time of sampling, leaving 34 patients for evaluation of *ex vivo* production of adipocytokine measurement and 38 patients for histological examination.

The Medical Ethics Committee approved the study. Written informed consent was obtained from each patient prior to study entrance. Cardiovascular history and blood pressure were obtained by the study physician. Blood was taken after an overnight fasting period.

PvAT biopsies and ex vivo incubation

The pvAT collection was performed right after implementation of the extra-corporal heart-lung system and administering of the cardioplegic fluid. Incision biopsies were obtained from adipose tissue surrounding the coronary arteries adjacent (<3mm) to a coronary artery segment with stenosis, as was determined before the surgical procedure, and to a non-stenotic coronary artery segment in the same patient. The selection of the sampling site was determined by the attending surgeon based on the coronary angiogram. For pvAT samples near stenotic coronary artery segments a stenosis of >50% was present. In all patients also control pvAT samples were taken near coronary artery segments without irregularities of the vascular wall. During surgery, the biopsy-site was carefully verified by palpation. In 27 (71%) cases the control pvAT sample was taken in the same coronary artery or its associated branch. In other cases the control pvAT sample was taken near another coronary artery in the same patients. The median weight of stenosis pvAT samples was 16 mg (IQR 9-35) and of control pvAT samples 10 mg (IQR: 6-19) (p=0.01).

Tissue biopsies from 34 patients were incubated in 500 µl serum-free Dulbecco's modified Eagles medium (DMEM) at 37°C. After 24 hours the supernatants from the pvAT cultures were centrifuged at 14,000 rounds per minute for 3 minutes. The centrifuged supernatants were stored at -80ºC until further processing.

Measurement of adipocytokines

Concentrations of adiponectin, cathepsin S, chemerin, soluble intercellular adhesion molecule-1 (sICAM-1), IL-1α, IL-5, IL-6, IL-13, IL-17, IL18, IL-23, IL-33, macrophage inflammatory protein 1α (MIP1α), macrophage colony stimulating factor (M-CSF), plasminogen activator inhibitor 1 (PAI-1), regulated upon activation normal T cell expressed and secreted (RANTES), retinol binding protein-4 (RBP-4), serum amyloid A1 (SAA-1), soluble vascular cell adhesion protein-1 (sVCAM-1), soluble vascular endothelial growth factor (sVEGF) and tissue inhibitor of metalloproteinases (TIMP-1) were measured in the undiluted supernatants with a multiplex assay (Biorad, Munich Gemany)[15]. The adipocytokines IL-1β, IL-2, IL-4, interferon-γ induced protein 10 (IP10), granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor (TNF)-α, were not detectable.

Histological examination

The pvAT samples from 38 patients were fixed in 10% formaldehyde and embedded in paraffin. Thin sections of 4 µm were processed for histological staining with hematoxylin & eosin (H&E). For quantification of macrophages (anti-CD68), lymphocytes (CD45) and capillary density (factor 8), immuno-histochemistry stainings with 3,3'-diaminobenzidine (DAB) were performed. M1 macrophages were defined as CD11c positive cells (Abcam monoclonal antibody EP1347Y) and M2 macrophages as CD206 positive cells (Abcam monoclonal antibody AB52632)[16]. Macrophages and lymphocytes were counted with the researcher blinded for the location of the biopsy. The mean number of positive staining cells in 3 high power fields was used for analysis (HPF; magnification 400x). The adipocyte size was determined by calculating the mean adipocyte area of 10 adipocytes in 10 randomly selected fields at 200x magnification[17]. Capillary density (%) was calculated by

measuring the area of von Willebrand factor-positive staining cells in 5 fields per slide at 200x magnification using Image pro plus software (Mediacybernetics).

Data analyses

Adipocytokine concentrations in the supernatants were corrected for sample weight. For IL-6, values that were above the detection limit (stenosis n=7, control n=3) were set as the upper limit of the range. Differences in adipocytokine concentration between stenosis and control pvAT samples were tested with Wilcoxon signed rank test. Taking multiple testing into account with Bonferroni correction for 23 adipocytokines, a p-value of <0.0021 instead of 0.05 was considered statistically significant. In addition, Wilcoxon signed rank test was used to test for differences in the number of macrophages and lymphocytes (n/HPF) and capillary density (%) between the two samples. A paired samples t-test was used to test differences in adipocyte size.

Beta coefficients with 95% confidence intervals (95%CI) were calculated for the relation between secreted adipocytokine levels and histological adipose tissue characteristics in stenosis and control pvAT samples. To ensure that the relation between adipocytokine concentrations and histological pvAT characteristics was linear, variables were logtransformed for the linear regression analyses. To test for differences in these relations between stenosis and control pvAT samples, interaction terms for the variables of the respective adipocytokines and sample site were entered in the model.

RESULTS

Patient characteristics

Patient characteristics are outlined in table 1. Mean age was 71 years and 27 out of 38 patients (71%) were male. Four patients (11%) had diabetes mellitus type 2. The reasons for surgery were aortic or mitral valve replacement in combination with CABG in 24 (63%) patients. In the other patients the reason for CABG was solely CAD. Fifteen (39%) patients had 3-vessel disease and 15 (40%) patients had 1-vessel disease.

Adipocytokine secretion

Ex vivo production of the pro-inflammatory adipocytokines IL-1α, IL-17, IL18 and IL-23 was higher in control pvAT compared to stenosis pvAT (p<0.0021; table 2). In addition, the anti-inflammatory cytokine IL-5 was increased in control pvAT compared to stenosis pvAT (difference -0.51; IQR -1.43- -0.07 p<0.0021). There were no adipocytokines produced at increased levels in stenosis versus control pvAT. There was a trend for higher secretion of IL-10, IL13, chemerin, M-CSF, MIP-1α, RANTES and SAA-1 in control pvAT compared to stenosis pvAT, but this was not statistically significant (p>0.0021).

Adipose tissue histology

Macrophages were more abundant in stenosis pvAT (2.3 n/HPF; IQR 0.3-4.5) than in control pvAT (1.2 n/HPF; IQR 0.1-2.5), whereas there was no difference in lymphocyte infiltration (figure 1). The median number of infiltrating M1 and M2 macrophages was low although M2 macrophages were more abundant than M1 macrophages (macrophages stenosis pvAT: M1: 0.0 /HPF; M2: 1.0 /HPF

Table 1 | Patient characteristics (n=38)

Age (years)	71±11
Male gender, n (%)	27 (71)
Current smoking, n (%)	9 (24)
Ever smoking, n(%)	23 (61)
Diabetes mellitus, n (%)	4 (11)
History of myocardial infarction, n (%)	3 (8)
Body mass index (kg/m²)	26.8±4.5
Waist circumference (cm)	100±14
Systolic blood pressure (mmHg)	133±16
Diastolic blood pressure (mmHg)	75±14
Blood-pressure lowering agents, n (%)	34 (90)
Statins, n (%)	29 (76)
Anti-platelet agents, n (%)	28 (74)
Fasting glucose (mmol/l)	5.6±2.1
Total cholesterol (mmol/l)	3.5±1.0
Triglycerides (mmol/l)	1.0±0.5
HDL-cholesterol (mmol/l)	0.95±0.37
LDL-cholesterol (mmol/l)	2.0±0.9
Fasting insulin (mIU/l)	5 (2-8)
hs-CRP (mg/l)	0.8 (0.5-3.7)
Creatinin clearance MDRD (ml/min/1.73m²)	125±45
1-vessel disease, n (%)	15 (40)
2-vesseldisease, n (%)	8 (21)
3-vesseldisease, n (%)	15 (39)
CABG alone	14 (37)
Aortic valve replacement n (%)	16 (42)
Mitral valve replacement / repair n (%)	8 (21)

p:0.004; macrophages control pvAT: M1 0.0/HPF; M2 0.6/ HPF p:0.013). The number of M1 and M2 macrophages did not differ between stenosis pvAT and control pvAT. Furthermore, adipocyte size did not differ between pvAT samples (stenosis: 3895±727 μm; control 3858±933 μm), while capillary density was more pronounced in stenosis pvAT (0.47% IQR 0.27-0.72) compared to control pvAT (0.33% IQR 0.24-0.52).

Ex vivo adipocytokine secretion in relation to perivascular adipose tissue histology

The relation between adipocytokine concentrations and histology was not different in stenosis pvAT and control pvAT (supplemental table 1, 2, 3). The pro- and anti-inflammatory adipocytokines IL-13, IL-33, IL-17, IL-23, chemerin, IL-1α and RANTES were negatively associated with macrophage

infiltration in stenosis pvAT (p<0.0021) (supplemental table 1). Lymphocyte infiltration was positively associated with cathepsin S in control pvAT. Furthermore, IL-6, sVCAM, sVEGF, cathepsin S and RBP4 increased with increasing CD206+ macrophages in stenosis pvAT (supplemental table 2). Relations between CD206+ macrophages and production of these cytokines did not significantly differ between stenosis and control pvAT. There was no relation between ex vivo adipocytokine production and adipocyte size or capillary density (supplemental table 3).

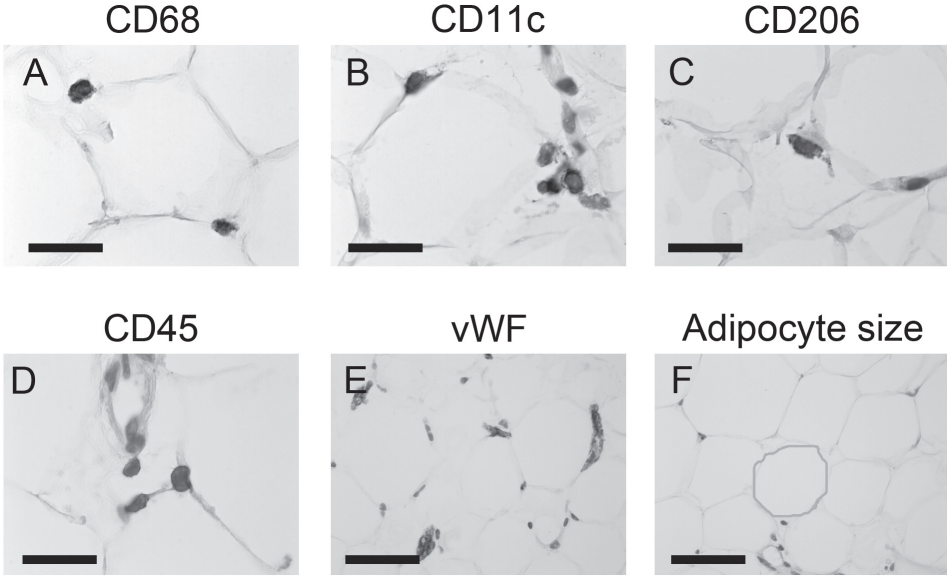
Table 2 | Concentrations of adipocytokines in perivascular adipose tissue near stenotic and non-stenotic coronary artery segments (n=34)

	pvAT Stenosis median (IQR)	**pvAT Control** median (IQR)	**Difference** median (IQR)
Anti-inflammatory adipocytokines			
Adiponectin (ng/ml/mg)	10.6 (6.1-33.9)	12.2 (0.6-15.1)	0.040 (-4.79-2.73)
IL-5 (pg/ml/mg)	0.63 (0.36-1.68)	1.51 (0.72-2.84)	-0.51 (-1.43- -0.07)*
IL-10 (pg/ml/mg)	1.35 (0.73-2.04)	1.94 (1.08-3.08)	-0.37 (-1.46-0.29)
IL-13 (pg/ml/mg)	0.73 (0.50-1.27)	1.05 (0.59-1.73)	-0.21 (-0.64-0.04)
IL-33 (pg/ml/mg)	2.48 (1.11-4.75)	3.58 (1.49-6.33)	-0.63 (-2.16-1.61)
Pro-inflammatory adipocytokines			
Chemerin (pg/ml/mg)	41.1 (26.1-79.1)	71.0 (41.2-124.1)	-24.1 (-63.2-2.3)
sICAM (pg/ml//mg)	228 (179-315)	284 (156-376)	-15.6 (-130.6-78.8)
IL-1a (pg/ml/mg)	0.82 (0.47-1.24)	1.31 (0.70-2.30)	-0.42 (-1.12-0.04)*
IL-6 (pg/ml/mg)	145 (50-313)	103 (40-266)	35 (-11-82)
IL-17 (pg/ml/mg)	4.22 (2.18-7.73)	7.40 (3.88-11.45)	-2.44 (-4.61- -0.39)*
IL-18 (pg/ml/mg)	0.51 (0.29-1.28)	1.10 (0.57-2.07)	-0.40 (-0.93- -0.04)*
IL-23 (pg/ml/mg)	58 (33-130)	113 (56-189)	-31 (-108- -5)*
Leptin (pg/ml/mg)	50.2 (38.6-94.6)	59.9 (37.0-108.4)	-11.1 (-30.0-26.8)
M-CSF (pg/ml/mg)	3.63 (2.08-5.47)	4.40 (2.55-7.92)	-0.88 (-4.42-1.20)
MIP1a (pg/ml/mg)	6.2 (3.6-8.9)	8.8 (4.3-12.4)	-1.7 (-6.9–2.0)
RANTES (pg/ml/mg)	39.2 (21.6-97.7)	68.3 (34.3-101.3)	-18.3 (40.0-11.7)
SAA-I (pg/ml/mg)	975 (641-1695)	1489 (821-2733)	-356 (-1226-79)
sVCAM (pg/ml/mg)	179 (94-323)	190 (111-302)	4 (-71-71)
sVEGF (pg/ml/mg)	14.8 (5.9-54.4)	12.9 (7.0-37.8)	1.0 (-4.4-10.3)
Other adipocytokines			
Cathepsin S (pg/ml/mg)	42.7 (18.9-62.9)	35.2 (21.7-65.5)	5.0 (-14.3-14.7)
PAI-1 (ng/ml/mg)	2.66 (1.46-3.58)	2.81 (1.72-5.12)	-0.44 (1.71-1.12)
RBP-4 (ng/ml/mg)	30.2 (15.2-42.8)	35.7 (21.0-41.7)	-0.9 (-1.2-6.0)
TIMP-1 (ng/ml/mg)	1.07 (0.34-1.79)	0.56 (0.23-1.27)	0.29 (-0.41-1.05)

*difference: p<0.0021

IQR: interquartile range; IL: interleukin; sICAM-1: soluble intercellular adhesion molecule-1; MIP-1α: macrophage inflammatory protein-1α, PAI-1: plasminogen activator inhibitor-1; RBP-4: retinol binding protein 4; RANTES: regulated upon activation normal T cell expressed and secreted; SAA-1: serum amyloid A1; sVCAM-1: soluble vascular cell adhesion protein-1; sVEGF: soluble vascular endothelial growth factor; TIMP-1: tissue inhibitor of metalloproteinases 1

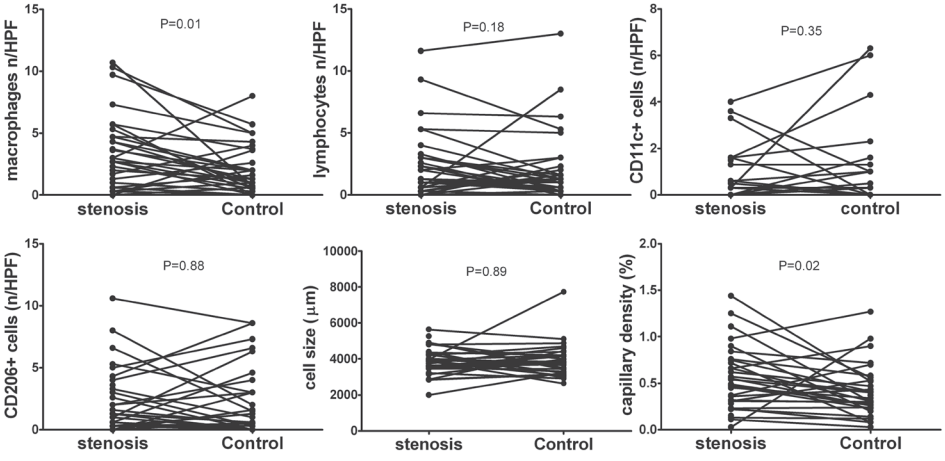
Figure 1 | Histological characteristics of perivascular adipose tissue (n=38).



a | Immunohistochemistry stainings

A: CD68 positive macrophages; B: M1 macrophages, CD11c positive; C: M2 macrophages, CD206 positive; D: lymphocytes, CD45 positive; E: endothelial cells, Factor 8/ von Willebrand factor (vWF) positive; F: adipocyte size indicated by the area within the green circumference. Bars indicate 20µm (A to D) or 100µm (E and F).

See page 148 for a full color representation of this figure



b | Number of macrophages and lymphocytes, capillary density and adipocyte size in perivascular adipose tissue near stenotic and non-stenotic (control) coronary artery segments

HPF: high power field 400x; M1 macrophages were defined as CD11c+ cells and M2 macrophages as CD206+ cells; stenosis: pvAT near stenotic coronary artery segments; control: pvAT near non-stenotic coronary artery segments.

DISCUSSION

Ex vivo production of the pro- and anti-inflammatory adipocytokines IL-5, IL-1α, IL17, IL18 and IL23 is higher in pvAT harvested near non-stenotic coronary artery segments compared to pvAT near stenotic coronary artery segments in patients undergoing CABG. Macrophages and capillaries were more abundant in pvAT samples near stenosis than in control pvAT samples and M2 macrophages were more abundant than M1 macrophages in pvAT near control and stenotic coronary artery segments.

The results of the present study indicate that there is a negative relation between adipocytokine production by pvAT and presence of local coronary atherosclerosis, in contrast to results from *in vitro* and animal studies. The positive results of clinical studies comparing the secretion pattern of CAD and non-CAD patients may be an overestimation of the paracrine effects of pvAT. In one of these studies, patients referred for CABG were 10 years older than non-CAD patients[12]. In addition, the use of statins and anti-platelet agents is typically lower in patients without CAD. Due to the small sample size of studies investigating epicardial adipose tissue, it is not possible to adjust for these factors in multivariable regression models. Therefore, the observed relation between secretion of adipocytokines by pvAT and the presence of CAD may partly be explained by confounding factors. Overall, epicardial adipose tissue of CAD patients has a more pro-inflammatory secretion pattern of adipocytokines than of non-CAD patients[12;18]. Besides lower adiponectin secretion there was a higher secretion of IL-6 and TNF-α by epicardial adipose tissue from CAD patients compared to epicardial adipose tissue from non-CAD patients[12;18;19]. Moreover, in a post mortem study, pvAT expression of adiponectin was negatively correlated with the severity of atherosclerosis in the adjacent coronary artery and in the aorta[20].

Difference in patient characteristics could not have influenced the results of the present study, because patients were their own control. PvAT samples were taken within the same patient. In a small study (n=11) in patients with CAD, adiponectin expression was lower in adipose tissue samples near a bare metal stent compared with samples near a coronary artery segment without stent, although this was not statistically significant[14]. In congruence with that study, we also found no differences in pvAT production of adiponectin between pvAT adjacent to coronary artery stenosis compared to control pvAT near non-stenotic coronary segments. The increased cytokine production in pvAT adjacent to non-stenotic coronary arteries contrasts with cytokine expression within plaques. IL-1 and IL-18 are produced at high levels in instable plaques[21;22], whereas it was secreted at lower levels in stenosis pvAT than by control pvAT in the current study. The role of cytokines may be different in coronary plaques compared to pvAT as illustrated by the role of CX3 chemokine receptor (CX3CR1) in macrophage recruitment. Although CX3CR1 is important in atherosclerotic plaque progression, CX3CR1 was not required for recruitment and retention of macrophages in murine adipose tissue[23].

Although the number of macrophages is more pronounced in stenosis pvAT compared to control pvAT samples, this is not accompanied by pro-inflammatory adipocytokine secretion. Furthermore, the ratio between M1 and M2 macrophages did not differ between stenosis pvAT and control pvAT. M1 macrophages are associated with a pro-inflammatory state and M2 macrophages with an anti-inflammatory state, secreting anti-inflammatory cytokines such as IL-4, IL-5 and IL-10[24]. Ex vivo secretion of these cytokines by pvAT was very low or even absent in the present study. Our observation of relatively low levels of CD11c+ M1 macrophages confirms the results of a study with epicardial

adipose tissue biopsies. In epicardial adipose tissue of CAD and non-CAD patients CD206+ M2 macrophages were more abundant than CD11c+ M1 macrophages[13]. It is possible that the predominance of M2 macrophages is a feature of desensitization of the inflammatory response in our study in patients with advanced CAD. Previously it was shown that the immune response is down regulated after arterial injury in vascular surgery[25]. In that study the TNF-α production in whole blood in response to LPS was markedly reduced after surgery. In contrast with our observation that there is no difference in the number of M1 and M2 macrophages in stenosis pvAT and control pvAT, macrophages are polarized towards a pro-inflammatory phenotype in patients with CAD as compared to patients without CAD[13].

We did not observe a difference in adipocyte size between stenosis pvAT and control pvAT samples in the present study, whereas macrophage infiltration and capillary density were more pronounced in stenosis pvAT samples. Cell size is a marker of tissue insulin resistance and adipose tissue dysfunction[26;27]. Cell size may not be a local marker for adipose tissue dysfunction in terms of adipocytokine production. A decrease in capillary density in adipose tissue, is generally thought to be related to adipose tissue inflammation[28]. Furthermore, obesity is associated with an increase in larger vessels in adipose tissue and with a decrease in capillaries in adipose tissue[4]. With expansion of visceral adipose tissue, the number of capillaries fails to meet the need for oxygen. In pvAT near stenosis, capillary density was higher than in pvAT near non-stenotic coronary artery segments. Hypoxia in pvAT near stenosis is not confirmed by increased sVEGF secretion. VEGF, which is induced by hypoxia and stimulates capillary formation[28], was secreted at equal levels by stenosis pvAT and control pvAT in our study.

Limitations of this study need to be considered. Importantly, this is a cross sectional study, meaning that we cannot draw conclusions concerning causality. Furthermore, pvAT samples near stenosis were on average larger than control pvAT samples. However, the conditions of sampling and handling pvAT harvested near stenotic and non-stenotic coronary artery segments were otherwise identical. The statistical power of the study is limited. It cannot be excluded that the number of adipocytokines that is secreted at different levels in stenosis and control pvAT is now underestimated. However, the methodological strength of the study was that pvAT biopsies were taken in the same patients from a stenotic and non-stenotic coronary artery segment, reducing the potential of confounding and enabling detailed evaluation of a local relation between pvAT and coronary atherosclerosis.

In conclusion, in patients with coronary artery disease, secretion of adipocytokines was higher in pvAT near non-stenotic than near stenotic coronary artery segments in patients undergoing CABG. Furthermore, pvAT macrophages but not M1 or M2 macrophages are associated with atherosclerosis of the adjacent vessel. The predominance of M2 macrophages in both samples may reflect desensitization of the inflammatory response in pvAT of patients with advanced CAD.

Competing interests

SV, MB, AV, LH, YG and FV declare: no financial relationships with any companies that might have an interest in the submitted work in the previous three years; and no other relationships or activities that could appear to have influenced the submitted work.

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REFERENCE LIST

1 Mazurek T, Zhang L, Zalewski A, Mannion JD, Diehl JT, Arafat H, et al. Human epicardial adipose tissue is a source of inflammatory mediators. Circulation 2003 Nov 18;108(20):2460-6.

2 Rajala MW, Scherer PE. Minireview: The adipocyte--at the crossroads of energy homeostasis, inflammation, and atherosclerosis. Endocrinology 2003 Sep;144(9):3765-73.

3 Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 2004 May;145(5):2273-82.

4 Spencer M, Unal R, Zhu B, Rasouli N, McGehee RE, Jr., Peterson CA, et al. Adipose Tissue Extracellular Matrix and Vascular Abnormalities in Obesity and Insulin Resistance. J Clin Endocrinol Metab 2011 Oct 12.

5 Qasim A, Mehta NN, Tadesse MG, Wolfe ML, Rhodes T, Girman C, et al. Adipokines, insulin resistance, and coronary artery calcification. J Am Coll Cardiol 2008 Jul 15;52(3):231-6.

6 Libby P, Okamoto Y, Rocha VZ, Folco E. Inflammation in atherosclerosis: transition from theory to practice. Circ J 2010 Feb;74(2):213-20.

7 Henrichot E, Juge-Aubry CE, Pernin A, Pache JC, Velebit V, Dayer JM, et al. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? Arterioscler Thromb Vasc Biol 2005 Dec;25(12):2594-9.

8 Verhagen SN, Visseren FL. Perivascular adipose tissue as a cause of atherosclerosis. Atherosclerosis 2011 Jan;214(1):3-10.

9 Miyata K, Shimokawa H, Kandabashi T, Higo T, Morishige K, Eto Y, et al. Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. Arterioscler Thromb Vasc Biol 2000 Nov;20(11):2351-8.

10 Shimokawa H, Ito A, Fukumoto Y, Kadokami T, Nakaike R, Sakata M, et al. Chronic treatment with interleukin-1 beta induces coronary intimal lesions and vasospastic responses in pigs in vivo. The role of platelet-derived growth factor. J Clin Invest 1996 Feb 1;97(3):769-76.

11 Ohman MK, Luo W, Wang H, Guo C, Abdallah W, Russo HM, et al. Perivascular visceral adipose tissue induces atherosclerosis in apolipoprotein E deficient mice. Atherosclerosis 2011 Jul 20.

12 Cheng KH, Chu CS, Lee KT, Lin TH, Hsieh CC, Chiu CC, et al. Adipocytokines and proinflammatory mediators from abdominal and epicardial adipose tissue in patients with coronary artery disease. Int J Obes (Lond) 2008 Feb;32(2):268-74.

13 Hirata Y, Tabata M, Kurobe H, Motoki T, Akaike M, Nishio C, et al. Coronary atherosclerosis is associated with macrophage polarization in epicardial adipose tissue. J Am Coll Cardiol 2011 Jul 12;58(3):248-55.

14 Spener RF, Breda JR, Pires AC, Pinhal MA, Souto RP. Adiponectin expression in epicardial adipose tissue after percutaneous coronary intervention with bare-metal stent. Rev Bras Cir Cardiovasc 2011 Sep;26(3):427-32.

15 Schipper HS, de JW, van Dijk ME, Meerding J, Zelissen PM, Adan RA, et al. A multiplex immunoassay for human adipokine profiling. Clin Chem 2010 Aug;56(8):1320-8.

16 Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007 Jan;117(1):175-84.

17 Faber DR, Moll FL, Vink A, van der Waal C, Kalkhoven E, Schipper HS, et al. Adipose tissue quantity and composition contribute to adipokine concentrations in the subclavian vein and the inferior mesenteric vein. Int J Obes (Lond) 2011 Nov 8.

18 Iacobellis G, Pistilli D, Gucciardo M, Leonetti F, Miraldi F, Brancaccio G, et al. Adiponectin expression in human epicardial adipose tissue in vivo is lower in patients with coronary artery disease. Cytokine 2005 Mar 21;29(6):251-5.

19 Zhou Y, Wei Y, Wang L, Wang X, Du X, Sun Z, et al. Decreased adiponectin and increased inflammation expression in epicardial adipose tissue in coronary artery disease. Cardiovasc Diabetol 2011;10(1):2.

20 Spiroglou SG, Kostopoulos CG, Varakis JN, Papadaki HH. Adipokines in periaortic and epicardial adipose tissue: differential expression and relation to atherosclerosis. J Atheroscler Thromb 2010 Feb 26;17(2):115-30.

21 Alexander MR, Moehle CW, Johnson JL, Yang Z, Lee JK, Jackson CL, et al. Genetic inactivation of IL-1 signaling enhances atherosclerotic plaque instability and reduces outward vessel remodeling in advanced atherosclerosis in mice. J Clin Invest 2012 Jan 3;122(1):70-9.

22 Mallat Z, Corbaz A, Scoazec A, Graber P, Alouani S, Esposito B, et al. Interleukin-18/interleukin-18 binding protein signaling modulates atherosclerotic lesion development and stability. Circ Res 2001 Sep 28;89(7):E41-E45.

23 Morris DL, Oatmen KE, Wang T, Delproposto JL, Lumeng CN. CX(3)CR1 Deficiency Does Not Influence Trafficking of Adipose Tissue Macrophages in Mice With Diet-Induced Obesity. Obesity (Silver Spring) 2012 Jan 17.

24 Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011 Nov 3;106(5):763-71.

25 Versteeg D, Dol E, Hoefer IE, Flier S, Buhre WF, de Kleijn D, et al. Toll-like receptor 2 and 4 response and expression on monocytes decrease rapidly in patients undergoing arterial surgery and are related to preoperative smoking. Shock 2009 Jan;31(1):21-7.

26 Maffeis C, Silvagni D, Bonadonna R, Grezzani A, Banzato C, Tato L. Fat cell size, insulin sensitivity, and inflammation in obese children. J Pediatr 2007 Dec;151(6):647-52.

27 Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. Diabetologia 2000 Dec;43(12):1498-506.

28 Pang C, Gao Z, Yin J, Zhang J, Jia W, Ye J. Macrophage infiltration into adipose tissue may promote angiogenesis for adipose tissue remodeling in obesity. Am J Physiol Endocrinol Metab 2008 Aug;295(2):E313-E322.

Supplemental table 1 | *Ex vivo* production of adipocytokines in relation to the number of macrophages and lymphocytes in stenosis and control pvAT

	Macrophages pvAT stenosis β (95%CI)	Macrophages pvAT no stenosis β (95%CI)	Lymphocytes pvAT stenosis β (95%CI)	Lymphocytes pvAT no stenosis β (95%CI)
Anti-inflammatory adipocytokines (pg/ml/mg)				
Adiponectin	-0.05 (-0.50-0.41)	0.04 (-0.17-0.24)	0.07 (-0.35-0.48)	0.19 (-0.03-0.41)
IL-5	-0.39 (-0.65- -0.14)	-0.20 (-0.44-0.05)	-0.26 (-0.51- -0.01)	-0.14 (-0.44- -0.17)
IL-10	-0.58 (-0.90- -0.27)	-0.25 (-0.53-0.04)	-0.38 (-0.70- -0.06)	-0.00 (-0.37-0.36)
IL-13	-0.59 (-0.90- -0.28)*	-0.05 (-0.26-0.17)	-0.21 (-0.54-0.13)	-0.03 (-0.28-0.22)
IL-33	-0.38 (-0.61- -0.15)*	-0.04 (-0.28-0.20)	-0.15 (-0.39-0.09)	0.04 (-0.23-0.31)
Pro-inflammatory cytokines (pg/ml/mg)				
Chemerin	-0.65 (-0.90- -0.40)*	-0.27 (0.54-0.00)	-0.37 (-0.65- -0.08)	-0.11 (-0.45-0.24)
sICAM	-0.39 (-0.74- -0.04)	-0.07 (-0.36-0.21)	0.15 (-0.49-0.19)	0.22 (-0.10-0.53)
IL-1a	-0.63 (-0.94- -0.31)*	-0.18 (-0.49-0.14)	-0.25 (-0.59-0.09)	0.06 (-0.32-0.43)
IL-6	0.16 (-0.07-0.38)	0.11 (-0.05-0.28)	0.18 (-0.03-0.38)	-0.32 (0.16-0.48*)
IL-17	-0.64 (-0.90- -0.38)*	-0.30 (-0.57- -0.04)	-0.34 (-0.62- -0.07)	-0.13 (-0.47-0.21)
IL-18	-0.34 (-0.59- -0.09)	-0.21 (-0.46-0.04)	-0.20 (-0.44-0.05)	-0.13 (-0.45-0.19)
IL-23	-0.48 (-0.72- -0.23)*	-0.26 (-0.50- -0.01)	-0.31 (-0.56- -0.06)	-0.16 (-0.47-0.15)
Leptin	-0.27 (-0.58-0.05)	-0.29 (-0.61-0.03)	0.09 (-0.22-0.39)	0.08 (-0.32-0.48)
M-CSF	-0.51 (-0.86- -0.17)	-0.33 (-0.63- -0.03)	-0.26 (-0.60-0.09)	-0.05 (-0.44-0.34)
MIP-1α	-0.51 (-0.86- -0.17)	-0.28 (-0.57-0.01)	-0.23 (-0.56-0.10)	-0.05 (-0.42-0.33)
RANTES	-0.62 (-0.84- -0.40)*	-0.27 (-0.54- -0.00)	-0.22 (-0.49-0.06)	-0.03 (-0.37-0.31)
SAA-1	-0.46 (-0.77- -0.16)	-0.09 (-0.32-0.15)	-0.26 (-0.57-0.04)	0.04 (-0.24-0.33)
sVCAM	-0.16 (-0.49-0.16)	-0.05 (-0.36-0.25)	0.08 (-0.22-0.38)	0.30 (-0.03-0.63)
sVEGF	0.13 (-0.08-0.35)	0.03 (-0.17-0.23)	0.14 (-0.06-0.33)	0.25 (0.04-0.45)
Other adipocytokines (pg/ml/mg)				
Cathepsin S	0.12 (-0.25-0.50)	0.21 (-0.07-0.48)	0.20 (-0.14-0.54)	0.50 (0.23-0.77)†
PAI-1	-0.02 (-0.42-0.38)	-0.02 (-0.23-0.26)	0.04 (-0.41-0.33)	0.12 (-0.17-0.40)
RBP4	-0.20 (-0.60-0.20)	-0.01 (-0.37-0.34)	0.16 (-0.21-0.53)	0.16 (-0.24-0.58)
TIMP-1	0.16 (-0.06-0.37)	0.13 (-0.02-0.28)	0.10 (-0.11-0.31)	0.24 (0.09-0.40)

adipocytokine concentrations and macrophages (n/HPF), lymphocytes (n/HPF) are logtransformed;
*: p<0.0021 (relation statistically significant)

Supplemental table 2 | Ex vivo production of adipocytokines in relation to the number of M1 and M2 macrophages in stenosis and control pvAT

	M1 Macrophages pvAT stenosis β (95%CI)	M1 Macrophages pvAT no stenosis β (95%CI)	M2 Macrophages pvAT stenosis β (95%CI)	M2 macrophages pvAT no stenosis β (95%CI)
Anti-inflammatory adipocytokines (pg/ml/mg)				
Adiponectin	-0.01 (-0.30-0.28)	0.11 (-0.06-0.27)	0.54 (0.16-0.92)	0.03 (-0.25-0.32)
IL-5	-0.12 (-0.30-0.06)	-0.07 (-0.27-0.13)	-0.28 (-0.54- -0.03)	-0.26 (-0.58-0.06)
IL-10	-0.17 (-0.41-0.06)	-0.11 (-0.36-0.13)	-0.07 (-0.43-0.29)	-0.09 (-0.51-0.33)
IL-13	-0.15 (-0.39-0.08)	-0.02 (-0.20-0.16)	-0.04 (-0.40-0.32)	-0.13 (-0.43-0.18)
IL-33	-0.13 (-0.29-0.03)	-0.05 (-0.24-0.15)	0.18 (-0.07-0.42)	0.07 (-0.25-0.39)
Pro-inflammatory cytokines (pg/ml/mg)				
Chemerin	-0.22 (-0.43- -0.02)	-0.14 (-0.38-0.09)	-0.13 (-0.46-0.19)	-0.18 (-0.58-0.22)
sICAM	-0.11 (-0.34-0.13)	0.04 (-0.19-0.29)	0.45 (0.14-0.77)	0.15 (-0.24-0.54)
IL-1a	-0.22 (-0.46-0.01)	-0.13 (-0.39-0.14)	0.03 (-0.34-0.39)	0.04 (-0.41-0.49)
IL-6	0.10 (-0.05-0.25)	0.10 (-0.04-0.24)	0.33 (0.13-0.52)*	0.28 (0.06-0.50)
IL-17	-0.21 (-0.42- -0.01)	-0.16 (-0.39-0.08)	-0.11 (-0.43-0.21)	-0.19 (-0.59-0.21)
IL-18	-0.08 (-0.25-0.09)	-0.08 (-0.28-0.13)	-0.26 (-0.50- -0.02)	-0.26 (-0.59-0.08)
IL-23	-0.13 (-0.31-0.05)	-0.12 (-0.33-0.08)	-0.25 (-0.52-0.02)	-0.28 (-0.62-0.07)
Leptin	-0.05 (-0.26-0.17)	-0.12 (-0.39-0.15)	0.35 (0.06-0.64)	0.05 (-0.42-0.51)
M-CSF	-0.13 (-0.37-0.12)	-0.11 (-0.38-0.15)	-0.07 (-0.44-0.30)	-0.19 (-0.64-0.26)
MIP-1α	-0.11 (-0.36-0.14)	-0.10 (-0.36-0.16)	0.10 (-0.26-0.46)	-0.13 (-0.56-0.30)
RANTES	-0.22 (-0.40- -0.03)	-0.09 (-0.32-0.15)	0.08 (-0.21-0.38)	-0.08 (-0.48-0.32)
SAA-1	-0.10 (-0.32-0.12)	-0.04 (-0.23-0.15)	-0.20 (-0.52-0.13)	-0.05 (-0.37-0.28)
sVCAM	-0.04 (-0.26-0.18)	0.02 (-0.23-0.27)	0.45 (0.19-0.72)*	0.22 (-0.18-0.62)
sVEGF	0.08 (-0.06-0.22)	0.03 (-0.15-0.20)	0.32 (0.15-0.49)*	0.24 (-0.03-0.50)
Other adipocytokines (pg/ml/mg)				
Cathepsin S	0.10 (-0.15-0.34)	0.20 (-0.03-0.44)	0.56 (0.26-0.85)*	0.40 (0.02-0.77)
PAI-1	-0.02 (-0.28-0.24)	-0.03 (-0.24-0.17)	0.22 (-0.16-0.60)	0.06 (-0.28-0.39)
RBP4	-0.07 (-0.33-0.20)	-0.13 (-0.44-0.18)	0.58 (0.24-0.91)*	0.18 (-0.31-0.67)
TIMP-1	0.05 (-0.10-0.20)	0.08 (-0.05-0.21)	0.19 (-0.02-0.40)	0.17 (-0.03-0.38)

M1 macrophages: CD11c positive cells (n/HPF); M2: M2 macrophages CD206 positive cells (n/HPF); adipocytokine concentrations and the number of macrophages are logtransformed;
*: $p<0.0021$ (relation statistically significant)

Supplemental table 3 | Ex vivo production of adipocytokines in relation to adipocyte size and capillary density in pvAT near stenosis and control pvAT

	Adipocyte size pvAT stenosis β (95%CI)	Adipocyte size pvAT no stenosis β (95%CI)	Capillary density pvAT stenosis β (95%CI)	Capillary density pvAT no stenosis β (95%CI)
Anti-inflammatory adipocytokines (pg/ml/mg)				
Adiponectin	-0.94 (-5.17-3.28)	-0.32 (-3.59-2.96)	0.09 (-0.40-0.58)	0.01 (-0.25-0.26)
IL-5	-2.84 (-5.3- -0.36)	-3.52 (-7.44-0.40)	0.01 (-0.31-0.33)	0.27 (-0.04-0.59)
IL-10	-2.00 (-5.51-1.50)	-4.54 (-9.21-0.12)	0.06 (-0.38-0.49)	0.23 (-0.17-0.64)
IL-13	-3.44 (-6.74- -0.14)	-2.07 (-5.43-1.31)	-0.01 (-0.41-0.40)	0.08 (-0.20-0.36)
IL-33	-1.52 (-3.96-0.92)	-2.99 (-6.70-0.72)	0.24 (-0.05-0.53)	0.02 (-0.28-0.32)
Pro-inflammatory cytokines (pg/ml/mg)				
Chemerin	-3.21 (-6.25- -0.18)	-4.87 (-9.24- -0.51)	0.05 (-0.33-0.42)	0.24 (-0.15-0.63)
sICAM	-0.32 (-3.79-3.15)	-4.94 (-9.38- -0.50)	0.11 (-0.30-0.53)	-0.07 (-0.44-0.30)
IL-1a	-3.86 (-7.18- -0.53)	-5.93 (-10.76- -1.09)	0.12 (-0.31-0.56)	0.22 (-0.20-0.64)
IL-6	0.87 (-1.33-3.06)	-0.06 (-3.00-2.88)	-0.01 (-0.27-0.25)	-0.04 (-0.26-0.18)
IL-17	-3.34 (-6.35- -0.34)	-5.27 (-9.56- -0.98)	0.06 (-0.31-0.44)	0.21 (-0.19-0.61)
IL-18	-2.51 (-4.89- -0.12)	-3.44 (-7.59-0.71)	0.01 (-0.30-0.32)	0.27 (-0.06-0.60)
IL-23	-2.79 (-5.36- -0.23)	-4.05 (-8.09- -0.01)	0.08 (-0.27-0.42)	0.31 (-0.02-0.64)
Leptin	1.13 (-1.98-4.23)	-1.42 (-7.08-4.24)	0.19 (-0.16-0.54)	0.21 (-0.25-0.66)
M-CSF	-3.29 (-6.72-0.16)	-4.65 (-9.78-0.46)	0.03 (-0.42-0.48)	0.27 (-0.16-0.70)
MIP-1α	-2.60 (-6.13-0.94)	-5.56 (-10.22- -0.91)	0.06 (-0.36-0.47)	0.24 (-0.18-0.67)
RANTES	-2.48 (-5.30-0.35)	-4.48 (-8.80- -0.16)	-0.08 (-0.27-0.42)	0.17 (-0.21-0.55)
SAA-1	-2.48 (-5.60-0.64)	-1.46 (-5.29-2.37)	0.08 (-0.27-0.42)	0.17 (-0.21-0.55)
sVCAM	1.53 (-1.50-4.57)	-2.92 (-7.94-2.11)	0.32 (-0.01-0.65)	0.21 (-0.18-0.60)
sVEGF	1.93 (-0.02-3.88)	-1.32 (-4.55-1.91)	-0.08 (-0.32-0.15)	-0.03 (-0.28-0.23)
Other adipocytokines (pg/ml/mg)				
Cathepsin S	0.44 (-3.17-4.05)	-3.03 (-7.99-1.93)	0.06 (-0.36-0.48)	-0.10 (-0.47-0.27)
PAI-1	0.19 (-3.62-3.99)	-0.59 (-4.46-3.29)	-0.03 (-0.46-0.41)	0.07 (-0.25-0.40)
RBP4	0.00 (-3.84-3.84)	-3.20 (-9.62-3.22)	0.13 (-0.32-0.58)	0.10 (-0.37-0.56)
TIMP-1	0.94 (-1.14-3.03)	0.06 (-2.46-2.57)	-0.04 (-0.29-0.21)	-0.02 (-0.22-0.18)

adipocytokine concentrations and capillary density (area %) are logtransformed;
*: p<0.0021 (relation statistically significant)

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Chapter 5

Polarized macrophages in perivascular adipose tissue and the adventitia in relation to features of instable atherosclerotic plaque in the coronary artery. A post-mortem study.

Submitted

ABSTRACT

Background: Perivascular adipose tissue (pvAT) may influence atherosclerotic plaque formation. We aim to determine the association between local macrophage polarization (M1 versus M2) in pvAT and features of instable atherosclerotic plaque (plaque macrophages and lipid core) in the left anterior descending artery (LAD).

Methods: Post mortem, 139 cross sections of the LAD were obtained from 16 patients. CD68+, CD11c+, CD206+ and CD163+ macrophages were determined in pvAT and adventitia. Generalized estimated equations analysis was performed to relate macrophage infiltration to quartiles of plaque macrophages (area (%)), and the presence of a lipid core (yes/no).

Results: CD11c+ macrophages were sparse in pvAT (median: 1.2 macrophages/mm^2) whereas pvAT was rich in CD206+ and CD163+ macrophages (median: 31.8 and 32.6 macrophages/mm^2 respectively). CD206+ macrophages (n/mm^2) in pvAT were related to plaque macrophages (OR per quartile: 1.02; 95%CI: 1.01-1.03; p=0.001), but CD11c+ macrophages(n/mm^2) in pvAT were not (OR per quartile: 1.07 95%CI:0.99-1.16; p=0.079). In congruence, CD206, but not CD11c macrophages were associated with the presence of a lipid core (CD206: median: 42.2/mm^2 versus 29.6/mm^2; p=0.006; CD11c:1.33/mm^2 versus 1.16/mm^2; p=0.58). Confirming observations of pvAT macrophage polarization, CD206+, but not CD11c+ macrophages in the adventitia (area%) were related to plaque macrophages or presence of a lipid core. The relations with CD206+ macrophages were confirmed with CD163+ macrophages.

Conclusion: In subjects with coronary atherosclerosis, CD11c+ M1-type macrophages are sparse in pvAT compared to M2-type macrophages. M2-type CD206+ and CD163+ macrophages in pvAT and the adventitia are related to plaque inflammation and the presence of a lipid core.

INTRODUCTION

In obesity the number of macrophages in adipose tissue increases, giving rise to a pro-inflammatory state of adipose tissue[1;2] contributing to systemic low-grade inflammation[3]. Systemic inflammation is acknowledged to play a causal role in the development of atherosclerosis[4]. Also local conditions such as non-laminar hemodynamic flow[5] and local inflammation[6;7] may contribute to atherosclerotic plaque development.

A phenotypic switch from the anti-inflammatory or alternatively activated M2 macrophage phenotype to the pro-inflammatory or classically activated M1 phenotype is observed in adipose tissue in obesity and also in atherosclerotic plaque[8;9]. It should be noted that the distinction between M1 and M2 macrophages is a simplification of the phenotypes that exist in vivo[9;10]. M1 macrophages are often described as CD11c expressing macrophages. The population of alternatively activated M2 macrophages is divers. Markers used to identify M2 macrophages are CD163 and CD206[11;12].

Local inflammation of perivascular adipose tissue (pvAT) is thought to influence the atherosclerotic process of the adjacent artery by local secretion of adipocytokines[13;14]. In animal studies, macrophages are attracted to the artery wall after application of chemotactic proteins to the outer vascular wall[15;16]. There have been a number of studies comparing characteristics of pvAT between patients, showing that secretion of pro-inflammatory cytokines an numbers of macrophages are higher in patients with coronary artery disease (CAD) than in patients without CAD[17;18]. However these results are likely to be subject to confounding as control subjects were less obese and were often younger in these studies[17;18]. Nevertheless, numbers of both CD11c+ and CD206+ macrophages were higher in epicardial adipose tissue of patients with CAD than in patients without CAD[12].

Instable atherosclerotic plaques are characterized by the presence of a large lipid core and by the infiltration of macrophages[19]. In these so called instable atherosclerotic lesions, on-going inflammation may lead to rupture of the fibrous cap and subsequent luminal thrombus formation. We previously showed that plaque macrophages in autopsy cases with severe coronary artery disease not only increase with the quantity of adventitia macrophages[20], but also with pvAT macrophages[21]. However, the phenotype of these macrophages has not yet been described.

The aim of the present post mortem study was to determine whether there is an association between CD11c, CD206 and CD163 macrophages in pvAT and the adventitia and features of atherosclerotic plaque instability.

METHODS

A total of 16 subjects with coronary atherosclerosis were included in the study between April 2009 and February 2011. Cases were referred for routine autopsy to the department of Pathology of the University Medical Centre in Utrecht the Netherlands. Eligible cases had a vascular cause of death or were >40 years of age. Moreover, they did not fulfil any of the exclusion criteria (history of PTCA, CABG, kidney failure, recent sepsis, known reduction of body weight (>5%) in the past 2 years, malignancy in the past 2 years, thyroid disease or oral use of steroid hormones). In 12 out of 16 cases death was caused by acute myocardial infarction (AMI) and a stenosis of the LAD was present in 6 out of the 12 AMI cases. In the other cases death was caused by aspiration pneumonia following ischemic stroke, by haemorrhagic stroke, ruptured aortic aneurysm and a work-related accident. The

study met the criteria of the code of proper use of human tissue that is used in the Netherlands for the use of human tissue.

Processing of coronary artery specimens

Bodies were stored at 4°C and autopsy was performed by the attending pathologist. The LAD was prepared from the heart with the surrounding adipose tissue and underlying myocardium taken along in a radius of 1-2 centimetres. The coronary arteries were fixed in formaldehyde 4% for one day. Subsequently the material was decalcified in ethylenediaminetetra-acetic acid (EDTA) solution during 2 to 5 days depending on the extent of calcification.

Coronary arteries were cross-sectioned, starting at the origin of the LAD (bifurcation of LAD and left circumflex coronary artery) to 6 cm from the origin, at 5 mm intervals (n=143). Subsequently, cross-sections where embedded in paraffin. Cross sections with an intra-myocardial localization of the LAD, referred to as myocardial bridge, were excluded because of virtual absence of adipose tissue (n=4 from 2 subjects). Per patient, a mean of 9±2 cross sections from 0.5 to 6.0 cm from the origin of the LAD were available, leaving 139 cross-sections for analyses.

Histology

Thin sections of 4 μm were processed for histological staining with hematoxylin & eosin (H&E), Picro-Sirius red, and elastin von Gieson. In addition immuno-histochemistry stainings were performed to quantify macrophages (CD68, CD11c, CD206 and CD163). Histology specimens were digitalized and analysed using Image Pro plus software (Mediacybernetics).

Macrophage infiltration in pvAT was scored by determining the mean number of cells with positive staining for CD68, CD11c, CD206 and CD163 respectively in 8 fields (magnification 200x). Fields were chosen in all quadrants around the coronary artery in the area next to the adventitia.

In the adventitia and atherosclerotic plaque it was not possible to count the number of individual macrophages, because macrophages were found in clusters. Therefore adventitia and plaque macrophages were quantified on digitalised slides of CD68, CD11c, CD206 and CD163 stainings respectively. The variable plaque macrophages was defined as the area (%) of the plaque occupied by CD68+ macrophages. To measure the area (%) of atherosclerotic plaques and the adventitia, 4 fields in all 4 quadrants of the intima and of the adventitia were chosen (magnification 200x)[22]. The presence of a lipid core was determined on H&E and Picro-Sirius red histology specimens.

Data analyses

As variables are skewed, distributions are presented as median with interquartile range (IQR). Analyses were performed with exclusion of outliers. Differences between the amount of different macrophage subtypes in pvAT (n/mm^2), the adventitia (area(%) and plaques (area(%)) were tested with Mann Whitney tests. The relation between macrophage subtypes in pvAT and quartiles of plaque macrophages (area (%) of CD68+ macrophages) was evaluated with ordinal logistic regression analyses. In addition, logistic regression general estimated equations (GEE) analyses were performed to determine the relation between macrophage subtypes in pvAT and the presence of a lipid core. In order to take clustering of characteristics within subjects into account, GEE analysis with a compound symmetry structure was used.

RESULTS

Patient characteristics

Mean age of the subjects was 63±13 years and 10 out of 16 subjects were male (63%). In 6 out of 12 subjects with AMI, the culprit lesion was located in the LAD. In the other 6 cases, the culprit lesion was located in de circumflex or right coronary artery. Type 2 diabetes was present in 3 cases and 50% of the individuals were current smokers.

Macrophages in pvAT, adventitia and atherosclerotic plaque

A median of 23.6 macrophages/mm^2 (IQR 8.4-42.0) positive for CD68 was present in pvAT. CD11c+ macrophages were sparse (median 1.2/mm^2; IQR 0.0-3.1), whereas CD206+ and CD163+ macrophages were more abundant (median 31.8/mm^2; IQR 17.5-45.5 and 32.6 /mm^2; IQR 19.8-50.7 respectively) (figure 1 and 2). In the adventitia, a median of 0.12% (IQR 0.05-0.32) of the area consisted of CD68+ macrophages and in the atherosclerotic plaque 0.18% (IQR 0.02-1.36). CD11+ macrophages occupied a median of 0.02% (IQR 0.00-0.06) in the adventitia whereas CD206+ macrophages occupied a median of 0.31% (IQR 0.19-0.60). In the atherosclerotic plaque, CD11c and CD206+ macrophages occupied 0.07% (IQR 0.01-0.67) and 0.15% (IQR 0.04-0.62) respectively. CD11c was mainly expressed by foam cells while CD206 staining was mainly observed in non-foamy macrophages. In adipose tissue a subset of CD11c+ macrophages stained positive for CD206 as well.

Macrophage subtypes in pvAT in relation to markers of instable atherosclerotic plaques

The number of CD206+ macrophages (n/mm^2) in pvAT increased with increasing quartiles of plaque macrophages (%) in the adjacent coronary artery (OR 1.02; 95%CI 1.01-1.03; p=0.001; figure 3). A relation between CD11c+ macrophages (n/mm^2) in pvAT and plaque macrophages could not be demonstrated, as it was borderline significant (OR 1.07 95%CI 0.99-1.16; p=0.08). In congruence, pvAT CD206+ macrophages (n/mm^2) were increased in plaques with a lipid core compared to plaques without a lipid core (median 42.2; IQR 27.8-68.9 versus 29.6; IQR 16.5-41.2, p<0.01), in contrast with CD11c+ macrophages (n/mm^2) (median: 1.33 IQR 0.00-3.50 versus 1.16 IQR 0.00-2.52 respectively; p=0.18).

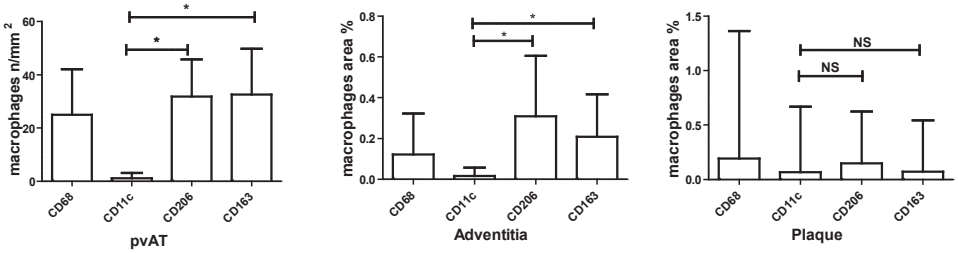

Figure 1: Macrophage subtypes in perivascular adipose tissue and atherosclerotic plaque.
*Bars represent median with interquartile range; *p<0.05; NS: not significant*

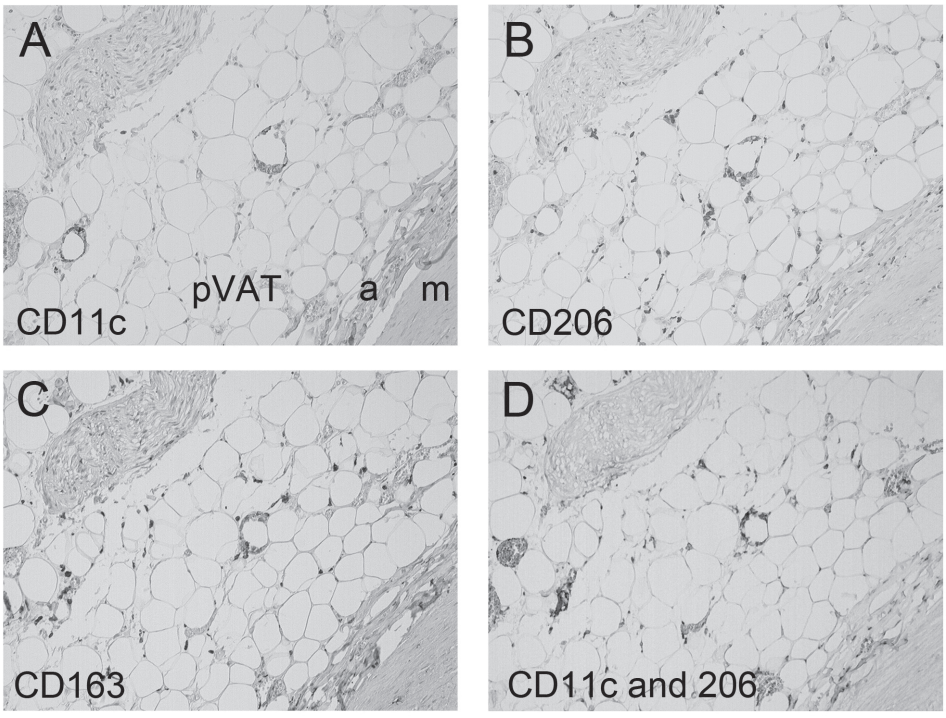

Figure 2: Immuno-histochemistry stainings of macrophage subtypes
Immuno-histochemistry stainings of M1 and M2 macrophages in coronary perivascular adipose tissue (pVAT). Consecutive sections: A: CD11c immuno-histochemistry staining showing some positive cells in pVAT. m, media; a, adventitia (magnification 100x). B and C: CD206 and CD163 immuno-histochemistry stainings showing abundant staining in pVAT and the adventitia. D: double immuno-histochemistry staining for CD11c and CD206.

See page 149 for a full color representation of this figure

Macrophage subtypes in the adventitia in relation to markers of instable atherosclerotic plaques

As in pvAT, the number CD206+ macrophages (n/mm^2) in the adventitia increased with plaque inflammation, measured as the area (%) occupied by CD68+ macrophages (OR 7.75; 95%CI 3.01-19.94; p<0.001). Adventitia CD11c+ macrophages (n*100/mm^2) were not demonstrably related to plaque inflammation (OR area %: 1.05; 95%CI 0.99-1.11 p=0.08), nor with the presence of a lipid core (lipid core: 0.04% IQR 0.01-0.18 versus no lipid core: 0.01%; IQR 0.00-0.04 p=0.178). CD206+ macrophages in the adventitia however, were more abundant in the presence of a lipid core (lipid core: 0.60% IQR 0.17-0.46 versus no lipid core: 0.59%; IQR 0.28-0.81 p<0.001).

The relation between CD163+ macrophages in pvAT and the adventitia and plaque inflammation and the presence of a lipid core confirmed the results retrieved for CD206+ macrophages (figure 3 and 4).

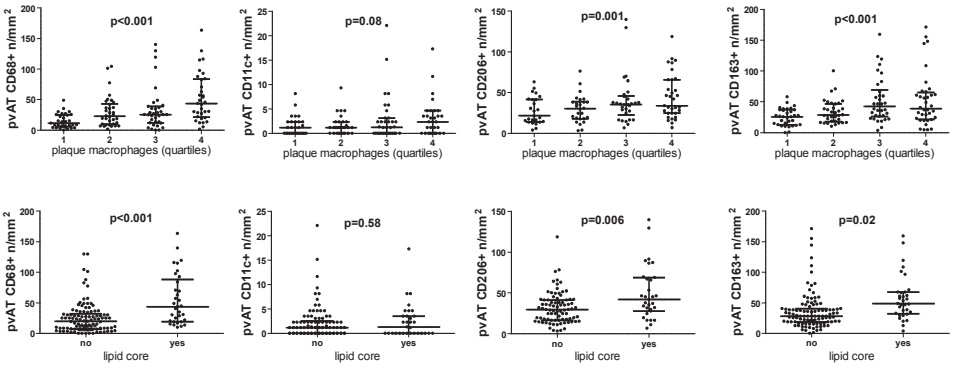

Figure 3: Macrophage subtypes in perivascular adipose tissue (pvAT) in relation to plaque macrophages and presence of a lipid core
Whiskers represent median with interquartile range

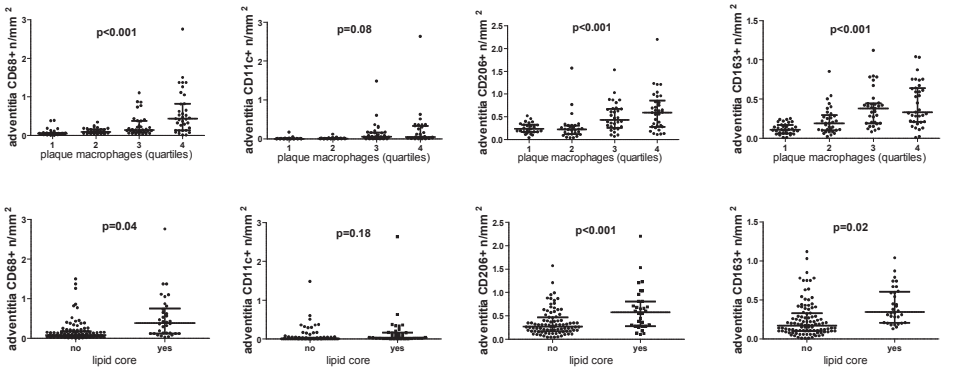

Figure 4: Macrophage subtypes in the adventitia in relation to plaque macrophages and presence of a lipid core
Whiskers represent median with interquartile range

DISCUSSION

Macrophage subtypes in pvAT are related to markers of atherosclerotic coronary plaque instability. Macrophages of the M1 type (CD11c+) were less abundant than M2 (CD206+ and CD163+) macrophages in coronary pvAT and adventitia. The amount of M2 macrophages in pvAT and the adventitia was associated with markers of instable atherosclerotic plaques, such as plaque inflammation and the presence of a lipid core, in the adjacent LAD cross section. In contrast with M1 macrophages in pvAT and the adventitia that were not related to the presence of a lipid core.

In visceral adipose tissue, an increase in CD11c+ macrophages is associated with obesity, hyperinsulinemia and upregulation of genes associated with the NFκB pathway[8;23]. Furthermore,

pro-inflammatory cytokine secretion in obese mice was reduced when CD11c+ macrophages were ablated[24]. This indicates that CD11c+ macrophages are involved in the induction of inflammatory reactions in adipose tissue. We did not observe a relation between local CD11c+ macrophages in pvAT and plaque inflammation, suggesting that the role of pro-inflammatory CD11c+ macrophages in pVAT on local plaque inflammation is limited. However, there was a relation between CD206 expressing macrophages in pvAT and plaque macrophages, possibly indicating the preponderance of anti-inflammatory stimuli in pvAT.

Our observations seem to contradict results of a recent case control study showing that both CD11c and CD206 expressing macrophages were higher in epicardial adipose tissue in subjects with coronary artery disease compared to controls[12]. In that study, the overall amount of CD11c+ macrophages in patients with atherosclerosis was higher than in controls. Adipose tissue samples were compared between patients, with the risk of inducing bias by confounding. However, we studied the local amount of pvAT macrophage subtypes within the same patient, observing that the local amount of CD11c+ macrophages was not associated with the local composition of the plaque. This suggests that the effect of M1 (CD11c+) macrophages on atherosclerotic plaques is rather a systemic than a local effect or that the differences in epicardial M1 macrophage infiltration between patients in the latter study were due to residual confounding.

M1 (CD11c+) macrophages in pvAT were not associated with instable plaques in the current study, although M1 macrophages are usually abundantly present in instable plaques[25;26]. The difference between pvAT and plaque macrophage polarization might be explained by the importance of the chemokine and chemokine receptors in trafficking of macrophages in atherosclerosis, but not in adipose tissue[27;28]. CX3CR1, for example, is not required for recruitment and retention of CD11c (M1) and CD206 (M2) expressing macrophages in murine adipose tissue, whereas CX3CR1 is important in atherosclerotic plaque progression[28]. Chemokine independent stimuli such as lipolysis or adipocyte necrotic adipocyte death may be involved in regulation of macrophage infiltration and polarization in pvAT[29;30].

Adventitial inflammation is known to accompany the presence and instability of atherosclerotic plaques[31]. Plaque inflammation and presence of a lipid core are associated with adventitial lymphocytic infiltrates in the human aorta[32] and coronary arteries[20]. In addition, in the latter study of acute coronary death cases[20], macrophage density in the intima is associated with presence of a lipid core of acute coronary death cases. We have now studied the subtypes of macrophages in the adventitia in relation to atherosclerotic plaques. Although more prominently present than in pvAT, M1 (CD11c+) macrophages were not related to markers of plaque instability, whereas M2 (CD206+ and CD163+) macrophages were increased in the adventitia of unstable plaques.

Polarized macrophages are not accurately described by dividing them in discrete subpopulations as macrophages are able to switch to a different phenotypic state when triggered by their environment[33]. Markers that are used to describe M1 macrophages are diverse. CD11c, which is highly associated with iNOS expression, is often used as a marker for M1 macrophages in histology[8;12]. The macrophage mannose receptor CD206 and the scavenger receptor CD163 are considered M2 markers[8;34]. In pvAT of rats there was only overlap in CD11c and CD206 expression in a small number of macrophages[35]. However, in the current study in human specimen the overlap in expression of these markers was commonly seen. Furthermore, in human atherosclerotic plaques there is an overlap in CD68, CD206 and CD163 expression[11]. Therefore the combination of CD11c and CD206 expressing macrophages is not always equal to the macrophages expressing the pan-macrophage marker CD68 in the current

study. The markers of choice (CD11c, CD206) are often used as markers for M1 and M2 polarized macrophages in human adipose tissue[9]. The fact that both used M2 markers in pvAT en adventitia were associated with features of plaque instability confirms that indeed these markers are representative for M2 macrophages.

A strength of our study is that macrophage subsets in perivascular adipose tissue were studied and related to plaque composition within patients. Study limitations need to be considered. Importantly, this is a cross sectional study, and therefore we are not able to make inferences about causality. It is unknown whether macrophage polarization in pvAT triggers atherosclerotic plaque development or whether it is the other way around. Moreover, we investigated several samples per subject which bears the risk of clustering of associations within subjects. Generalised estimated equations with a compound symmetry structure were performed in order to account for correlations between multiple observations within a subject.

In conclusion, in patients with coronary atherosclerosis expression of CD206 and CD163 in pvAT and adventitia is related to local plaque inflammation and the presence of a lipid core of the adjacent coronary artery. In contrast, CD11c macrophages are sparse in pvAT and are not associated with the presence of a lipid core. These results suggest that the association between pvAT macrophages with markers of coronary artery plaque instability is determined by M2 macrophages.

REFERENCE LIST

1 Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 2008; 29(24):2959-2971.

2 Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003; 112(12):1796-1808.

3 Faber DR, van der Graaf Y, Westerink J, Visseren FL. Increased visceral adipose tissue mass is associated with increased C-reactive protein in patients with manifest vascular diseases. Atherosclerosis 2010; 212(1):274-280.

4 Ross R. Atherosclerosis--an inflammatory disease. N Engl J Med 1999; 340(2):115-126.

5 Shinjo SK, Prates NE, Oba SM, Sampaio LO, Nader HB. Distribution and composition of glycosaminoglycans in the left human coronary arterial branches under myocardial bridge. Atherosclerosis 1999; 143(2):363-368.

6 Pasterkamp G, Schoneveld AH, van der Wal AC, Hijnen DJ, van Wolveren WJ, Plomp S et al. Inflammation of the atherosclerotic cap and shoulder of the plaque is a common and locally observed feature in unruptured plaques of femoral and coronary arteries. Arterioscler Thromb Vasc Biol 1999; 19(1):54-58.

7 Vink A, Schoneveld AH, Richard W, de Kleijn DP, Falk E, Borst C et al. Plaque burden, arterial remodeling and plaque vulnerability: determined by systemic factors? J Am Coll Cardiol 2001; 38(3):718-723.

8 Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007; 117(1):175-184.

9 Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011; 106(5):763-771.

10 Kadl A, Meher AK, Sharma PR, Lee MY, Doran AC, Johnstone SR et al. Identification of a novel macrophage phenotype that develops in response to atherogenic phospholipids via Nrf2. Circ Res 2010; 107(6):737-746.

11 Finn AV, Nakano M, Polavarapu R, Karmali V, Saeed O, Zhao X et al. Hemoglobin directs macrophage differentiation and prevents foam cell formation in human atherosclerotic plaques. J Am Coll Cardiol 2012; 59(2):166-177.

12 Hirata Y, Tabata M, Kurobe H, Motoki T, Akaike M, Nishio C et al. Coronary atherosclerosis is associated with macrophage polarization in epicardial adipose tissue. J Am Coll Cardiol 2011; 58(3):248-255.

13 Sacks HS, Fain JN. Human epicardial adipose tissue: a review. Am Heart J 2007; 153(6):907-917.

14 Verhagen SN, Visseren FL. Perivascular adipose tissue as a cause of atherosclerosis. Atherosclerosis 2011; 214(1):3-10.

15 Miyata K, Shimokawa H, Kandabashi T, Higo T, Morishige K, Eto Y et al. Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. Arterioscler Thromb Vasc Biol 2000; 20(11):2351-2358.

16 Shimokawa H, Ito A, Fukumoto Y, Kadokami T, Nakaike R, Sakata M et al. Chronic treatment with interleukin-1 beta induces coronary intimal lesions and vasospastic responses in pigs in vivo. The role of platelet-derived growth factor. J Clin Invest 1996; 97(3):769-776.

17 Cheng KH, Chu CS, Lee KT, Lin TH, Hsieh CC, Chiu CC et al. Adipocytokines and proinflammatory mediators from abdominal and epicardial adipose tissue in patients with coronary artery disease. Int J Obes (Lond) 2008; 32(2):268-274.

18 Iacobellis G, Pistilli D, Gucciardo M, Leonetti F, Miraldi F, Brancaccio G et al. Adiponectin expression in human epicardial adipose tissue in vivo is lower in patients with coronary artery disease. Cytokine 2005; 29(6):251-255.

19 Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. Arterioscler Thromb Vasc Biol 2000; 20(5):1262-1275.

20 Tavora F, Kutys R, Li L, Ripple M, Fowler D, Burke A. Adventitial lymphocytic inflammation in human coronary arteries with intimal atherosclerosis. Cardiovasc Pathol 2010; 19(3):e61-e68.

21 Verhagen SN, Vink A, Graaf Y, Visseren FL. Coronary perivascular adipose tissue characteristics are related to atherosclerotic plaque size and composition. A post-mortem study. In revision.

22 Hellings WE, Pasterkamp G, Vollebregt A, Seldenrijk CA, de Vries JP, Velema E et al. Intraobserver and interobserver variability and spatial differences in histologic examination of carotid endarterectomy specimens. J Vasc Surg 2007; 46(6):1147-1154.

23 Le KA, Mahurkar S, Alderete TL, Hasson RE, Adam TC, Kim JS et al. Subcutaneous adipose tissue macrophage infiltration is associated with hepatic and visceral fat deposition, hyperinsulinemia, and stimulation of NF-kappaB stress pathway. Diabetes 2011; 60(11):2802-2809.

24 Patsouris D, Li PP, Thapar D, Chapman J, Olefsky JM, Neels JG. Ablation of CD11c-positive cells normalizes insulin sensitivity in obese insulin resistant animals. Cell Metab 2008; 8(4):301-309.

25 Boyle JJ, Harrington HA, Piper E, Elderfield K, Stark J, Landis RC et al. Coronary intraplaque hemorrhage evokes a novel atheroprotective macrophage phenotype. Am J Pathol 2009; 174(3):1097-1108.

26 Chinetti-Gbaguidi G, Baron M, Bouhlel MA, Vanhoutte J, Copin C, Sebti Y et al. Human atherosclerotic plaque alternative macrophages display low cholesterol handling but high phagocytosis because of distinct activities of the PPARgamma and LXRalpha pathways. Circ Res 2011; 108(8):985-995.

27 Kirk EA, Sagawa ZK, McDonald TO, O'Brien KD, Heinecke JW. Monocyte chemoattractant protein deficiency fails to restrain macrophage infiltration into adipose tissue [corrected]. Diabetes 2008; 57(5):1254-1261.

28 Morris DL, Oatmen KE, Wang T, Delproposto JL, Lumeng CN. CX(3)CR1 Deficiency Does Not Influence Trafficking of Adipose Tissue Macrophages in Mice With Diet-Induced Obesity. Obesity (Silver Spring) 2012.

29 Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 2005; 46(11):2347-2355.

30 Kosteli A, Sugaru E, Haemmerle G, Martin JF, Lei J, Zechner R et al. Weight loss and lipolysis promote a dynamic immune response in murine adipose tissue. J Clin Invest 2010; 120(10):3466-3479.

31 Maiellaro K, Taylor WR. The role of the adventitia in vascular inflammation. Cardiovasc Res 2007; 75(4):640-648.

32 Moreno PR, Purushothaman KR, Fuster V, O'Connor WN. Intimomedial interface damage and adventitial inflammation is increased beneath disrupted atherosclerosis in the aorta: implications for plaque vulnerability. Circulation 2002; 105(21):2504-2511.

33 Kawanishi N, Yano H, Yokogawa Y, Suzuki K. Exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages in high-fat-diet-induced obese mice. Exerc Immunol Rev 2010; 16:105-118.

34 Bouhlel MA, Derudas B, Rigamonti E, Dievart R, Brozek J, Haulon S et al. PPARgamma activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. Cell Metab 2007; 6(2):137-143.

35 Zeyda M, Gollinger K, Kriehuber E, Kiefer FW, Neuhofer A, Stulnig TM. Newly identified adipose tissue macrophage populations in obesity with distinct chemokine and chemokine receptor expression. Int J Obes (Lond) 2010; 34(12):1684-1694.

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Chapter 6

Relationship between myocardial bridges and reduced coronary atherosclerosis in patients with angina pectoris

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ABSTRACT

Background: A myocardial bridge (MB) is a band of myocardium covering a coronary artery segment, typically located in the left anterior descending coronary artery (LAD). Bridged segments of the coronary artery are isolated from the influence of perivascular adipose tissue. The aims of this study were to investigate the relationship between MBs and atherosclerosis in bridged LAD segments and to evaluate whether perivascular adipose tissue is involved in this relationship.

Methods: MBs were identified in the coronary arteries of patients referred for diagnostic cardiac CT. The calcium score of MBs of the LAD or, in patients without LAD-MBs, of a corresponding LAD segment at the same distance from its origin and over the same length was measured.

Results: Of 128 patients, 56 (44%) had in total 73 MBs. The mean MB length was 22±14 mm and the median MB thickness was 0.8 mm (interquartile range 0.3–2.1 mm). MBs in the LAD were present in 40 patients (31%). The calcium score was 0 in 95% of the LAD segments with MBs compared with 52% of the corresponding LAD segments without MBs. The association between LAD-MBs and calcium score (OR 0.06, 95% CI 0.01–0.25) was not influenced by age and gender, but was attenuated by local perivascular adipose tissue thickness (OR 0.35, 95% CI 0.04–2.70).

Conclusions: Coronary artery segments covered with an MB have a lower calcium score than segments without an MB. The association between MBs and calcium scores was influenced by local perivascular adipose tissue thickness.

INTRODUCTION

The coronary arteries usually run an epicardial course and are surrounded by coronary perivascular adipose tissue. A variant of coronary artery anatomy is the myocardial bridge (MB), whereby part of the coronary artery runs through the myocardium. The reported prevalence of MBs varies between 5% and 86%.[1-3] MBs are most common in the left anterior descending artery (LAD).[2] Interestingly, coronary artery segments with MBs have been found to be free of atherosclerotic plaque in autopsy [3-5] and CT[6-9] studies, although in the CT studies no comparable control groups or control segments were investigated. Hemodynamic factors have been proposed to explain the absence of atherosclerosis in bridged coronary artery segments [10], but an alternative view is that bridged coronary arteries are protected from the influence of perivascular adipose tissue by the overlying myocardium.

Most arteries are directly surrounded by perivascular adipose tissue, and the diffusion of pro-inflammatory cytokines and adipokines may locally affect the metabolism of the coronary artery wall by inducing endothelial dysfunction, monocyte chemotaxis, and smooth muscle cell proliferation[11-13]. Indeed, it could be hypothesized that perivascular adipose tissue stimulates atherosclerotic plaque formation from outside to inside. Abdominal obesity is a risk factor for the development of cardiovascular diseases by inducing systemic metabolic disturbances, such as inflammation, insulin resistance, dyslipidemia, and hypercoagulability[14]. This is thought to be a consequence of adipose tissue dysfunction, which is characterized by the secretion of pro-inflammatory cytokines and adipokines, such as tumor necrosis factor-α (TNF- α), interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1) and the inhibitor of fibrinolysis plasminogen activator inhibitor-1 (PAI-1)[15].

We hypothesized that sections of coronary artery covered by an MB do not show atherosclerotic changes and that this might be due to the lack of exposure to perivascular adipose tissue at these sites. Therefore, the aims of the present study were to compare the extent of atherosclerosis, measured as coronary calcium, in coronary artery segments with or without MBs in patients referred for angina pectoris, and to investigate the potential role of perivascular adipose tissue in this relationship.

METHODS

Study participants and design

Consecutive patients (n=128) referred to the University Medical Center Utrecht, the Netherlands, for diagnostic coronary angiography for stable or unstable angina pectoris were included in this cross-sectional study. Inclusion criteria were age 50-70 years and stable sinus rhythm. Patients who had previously undergone PTCA or CABG, or who had serum creatinine levels >140 μmol/l or known iodine-based contrast allergy were excluded. The medical ethics committee approved the study and all participants gave their written informed consent.

Medical history was obtained using a standardized health questionnaire. Height, weight, blood pressure, and the volume of epicardial adipose tissue[16] were measured. Epicardial adipose tissue volume was defined as the adipose tissue between the ventricular wall and the visceral epicardium. Fasting blood was sampled to measure lipid, glucose, and creatinine levels. Metabolic syndrome was diagnosed according to the Adult Treatment Panel III (ATPIII) criteria as the presence of at least three or more metabolic abnormalities.[17] Because waist circumference was not available, a BMI ≥30

kg/m^2 was considered indicative of obesity.[18] The number of coronary arteries (RCA, LAD, and RCX) with ≥50% stenosis was evaluated on multiple conventional coronary angiography projections to determine whether subjects had 0, 1, 2, or 3-vessel disease.[19] Left main artery stenosis was scored as 2-vessel disease[19].

CT technique and image analysis

MBs were identified on retrospectively ECG-gated diagnostic cardiac CT scans acquired with a 64 detector-row CT scanner (Brilliance 64, Philips Medical Systems, Cleveland, OH, USA). Scan duration was 7-10 seconds. Standard coronary CT angiography protocols were used, including the use of intravenous beta-blockers for patients with a heart rate >65 beats/min (unless contraindicated), and images were acquired when the subject held his/her breath during inspiration. Imaging parameters were slice collimation of 64 x 0.625 mm, gantry rotation time of 420 ms, tube voltage of 120 kVp, tube current of 900 mAs. The contrast agent iopromide (Bayer-Schering AG, Berlin, Germany) was injected intravenously (1.6-2.0 g iodine/s depending on patient's body weight). The most motionless phase of the cardiac cycle, usually at 70-80% of the R-R interval, was chosen for analysis. The window settings were adjusted to enable optimal visualization of soft tissue.

Identification and measurement of MBs

MBs, defined as a coronary artery segment covered by myocardium, were detected on contrast-enhanced scans. Although limited spatial resolution makes it difficult to visualize MBs <0.3 mm thick,[20] we included these bridges in the analyses, because the absence of atherosclerosis in thin MBs would be strong evidence in favour of our hypothesis. Measurements were performed on curved planar reformation views. The length of the MB was defined as the length of the intramyocardial course of the coronary artery segment. The thickness of an MB was measured at the widest part of the overlying myocardium (figure 1). If the thickness of a MB could not be measured (<0.3 mm), it was given the value of 0.3 mm.

Measurement of calcium score in coronary artery segments

Coronary artery calcium imaging was performed using multi-slice CT scanning. Non-contrast scans, reconstructed with 3-mm-slice thickness and an increment of 1.5 mm, were acquired during a single breath-hold. The Agatston calcium score was chosen as measure of atherosclerosis and was calculated with dedicated software for calcium scoring (ImageXplorer)[21]. For total calcium score, all regions with a density >130 HU and a minimum size of 0.5 mm^2 were identified as potential calcifications. The peak density in HU and the area in mm^2 in each selected region were calculated and multiplied by a weighting factor[22].

The calcium score within the LAD was also calculated. To compare coronary artery segments with and without MBs, an area of interest was selected for each subject. Subjects with LAD-MBs (n=40) were randomly paired with subjects without LAD-MBs (n=88). The calcium score of the intramyocardial artery segment was measured in patients with a LAD-MB and in a section of the LAD at the same distance from the origin of the left coronary artery and over the same length in subjects without a LAD-MB (figure 1). In addition, the presence of atherosclerotic plaque in the same bridged and matched LAD segments was determined.

Measurement of perivascular adipose tissue thickness

The thickness of perivascular adipose tissue was measured in the region of interest in the LAD as the mean of two measurements from the myocardium to the pericardium at the site of minimum and maximum thickness, respectively. Measurements where performed on curved planar reformation views. Segments with MBs were not in contact with perivascular adipose tissue.

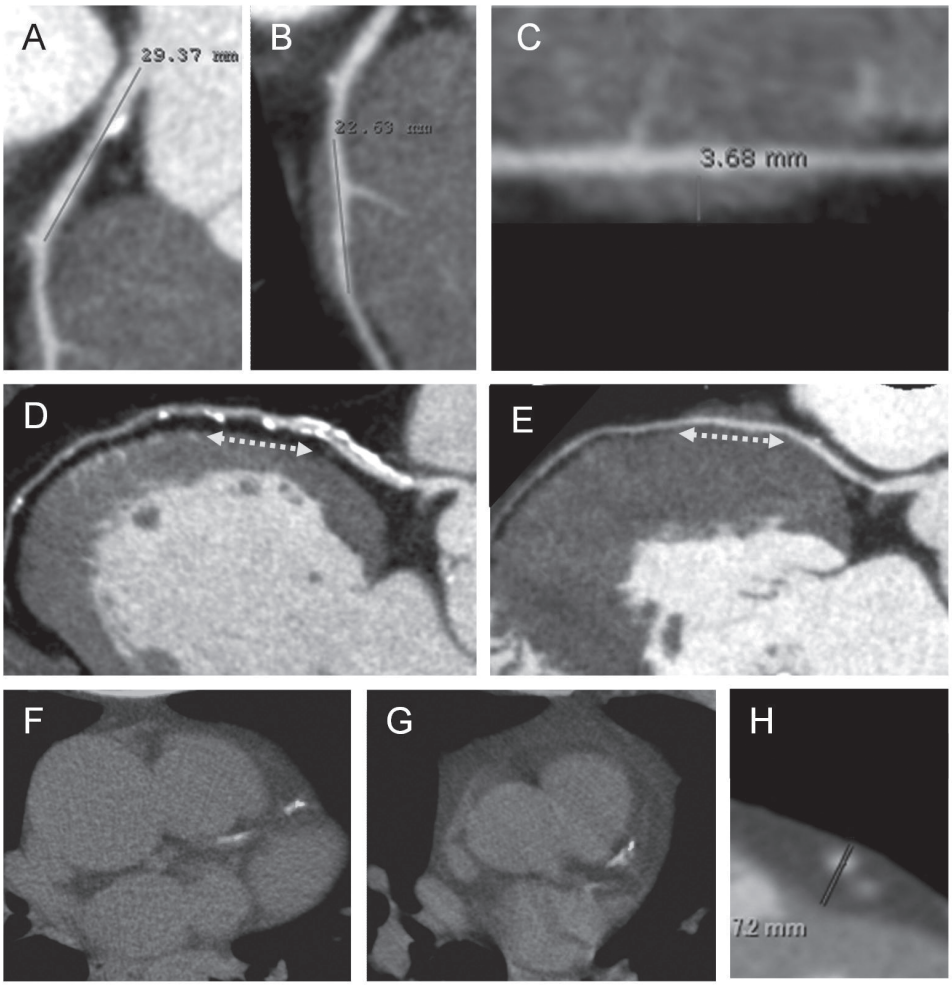

Figure 1 | Detection of myocardial bridges (MBs), measurement of MB length and thickness, and measurement of calcium score of coronary artery segments with and without an MB.
The following MB properties were measured: distance to origin (A), length (B), and thickness (C). Calcium score was measured in bridged coronary artery segments and corresponding segments in subjects without an MB. In subjects without an MB (D), calcium score was measured in a segment at the same distance to origin and over the same length as in subjects with an MB (E). (Selected regions are indicated with an arrow). Calcium score was measured in the region of interest (F) (G). Perivascular adipose tissue thickness was measured in the regions of interest (H).

See page 150 for a full color representation of this figure

Data analyses

Analyses were performed with SPSS 18. Associations between the presence of an MB (yes/no) and age, gender, and cardiometabolic risk factors were analysed with logistic regression analysis. Differences in the length and thickness of an MB with respect to its location were calculated and tested with Kruskal-Wallis test.

Differences in the calcium score of bridged and matched segments (0 versus >0) in patients with or without MBs were compared and significance was tested with Chi-square. The relationship between MBs and calcium scores in the region of interest was analysed with univariate and multivariate logistic regression modelling. The calcium score in the region of interest (0 or >0) was used as dependent variable and the presence of an MB (yes/no) was used as independent variable. Different models were constructed to adjust for potential confounding factors, such as age and gender (model 2), calcium score (model 3), and local perivascular adipose tissue thickness (model 4).

In patients without MBs, perivascular adipose tissue was used as independent variable in linear regression analyses with local calcium score as outcome. Calcium scores were log-transformed (LN(calcium score +1) because scores were not normally distributed in the regions of interest.

The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology[23].

RESULTS

Baseline characteristics of study population

In 56 of the 128 subjects (44%) 73 MBs were detected, and 44 of these MBs (in 43 subjects) had a thickness ≥0.3 mm. In 40 (31%) subjects MBs of the LAD were detected, and 27 of these MBs had a thickness ≥0.3 mm.

The characteristics of patients with (n=40) and without (n=88) a LAD-MB were comparable (table 1), except that patients with a LAD-MB had more severe coronary artery disease, as determined with conventional angiography, with ≥2 vessel disease being detected in 23 patients with a LAD-MB (58%) compared with 32 patients without a LAD-MB (36%) (p=0.03). Total calcium scores were not statistically significantly different between the groups with or without a LAD-MB (median 135, interquartile range 35-622 versus median 184, IQR 45-502, respectively; p=0.76). In addition, the prevalence of LAD stenosis ≥50% was similar in the two groups, occurring in 21 (53%) and 46 (53%) patients with and without a LAD-MB, respectively (p=0.98). The same was true for the LAD calcium score: median score 61 (IQR 19-225) versus 107 (IQR 15-242), respectively (p=0.35). The mean length of LAD-MBs was 21.7±14.9 mm and the median thickness was 0.9 mm (IQR 0.3-2.2 mm) (figure 2). The mean length of diagonal branch MBs was 19.8±10.2 mm with a median thickness of 0.8 mm (IQR 0.3-2.1 mm), and the mean length of marginal obtusis MBs was 24.0±14.0 mm with a median thickness of 0.9 mm (IQR 0.1-1.7 mm). There were no statistically significant differences in the length and thickness of MBs in the LAD, diagonal branch, or marginal obtusis branch (p=0.8 and p=0.5 respectively).

Relation between LAD-MBs and local atherosclerosis

Out of the 40 subjects with a LAD-MB 2 (5%) had a calcium score >0 in the bridged region, whereas 42 of the 88 subjects without an LAD-MB (48%) had a calcium score >0 in the matched LAD

Table 1 | Patient characteristics

	All cases N = 128	No (LAD) MB N = 88	(LAD) MB N = 40
Age (years)	61 ± 6	61 ± 5	61 ± 6
Male gender, n (%)	89 (70)	59 (67)	30 (75)
Current smoking, n (%)	42 (33)	29 (33)	13 (33)
Diabetes mellitus, n (%)	24 (19)	17 (19)	7 (18)
Stable angina pectoris, n (%)	100 (78)	68 (77)	32 (80)
History of unstable angina pectoris, n (%)	8 (6)	5 (6)	3 (8)
History of myocardial infarction, n (%)	29 (23)	21 (24)	8 (20)
Body mass index (kg/m^2)	28 ± 4	28 ± 4	27 ± 4
Systolic blood pressure (mmHg)	151 ± 22	151 ± 22	152 ± 24
Diastolic blood pressure (mmHg)	81 ± 13	81 ± 12	81 ± 15
Glucose (mmol/l)	5.8 (5.2-6.8)	5.8 (5.2-6.9)	5.8 (5.1-6.6)
Total cholesterol (mmol/l)	4.6 ± 1.2	4.5 ± 1.1	4.9 ± 1.2
LDL-cholesterol (mmol/l)	2.6 ± 1.0	2.5 ± 1.0	2.7 ± 1.1
Triglycerides (mmol/l)	1.5 (1.1-2.1)	1.5 (1.0-2.1)	1.4 (1.1-2.4)
HDL-cholesterol (mmol/l)	1.3 ± 0.3	1.3 ± 0.3	1.3 ± 0.4
Metabolic syndrome n (%)	55 (43)	37 (42)	18 (45)
Blood-pressure lowering agents, n (%)	115 (90)	78 (89)	37 (93)
Lipid-lowering agents, n (%)	100 (78)	65 (74)	35 (88)
0 vessel disease	19 (15)	14 (16)	5 (13)
1-vessel disease	54 (42)	42 (48)	12 (30)
2-vessel disease	37 (29)	20 (23)	17 (43)
3-vessel disease	18 (14)	12 (14)	6 (15)
Stenosis >50% of the LAD, n (%)	67 (52)	46 (52)	21 (53)
Epicardial adipose tissue volume (cm^3)	110 ± 44	113 ± 46	103 ± 39
Local perivascular adipose tissue (mm)	-	9.0 ± 3.2	0
Calcium score of the LAD (Agatston)	92 (16-232)	107 (15-242)	61 (19-225)
Total calcium score (Agatston)	181 (37-544)	184 (35-622)	135 (45-502)
Presence of MB:			
No MB	72 (56)	72 (82)	0 (0)
MB (LAD), n (%)	40 (31)	0 (0)	40 (100)
MB (1st diagonal branch), n (%)	5 (3)	2 (2)	3 (5)
MB (2nd diagonal branch), n (%)	4 (2)	3 (3)	1 (3)
MB (1st marginal obtusis branch), n (%)	13 (10)	9 (10)	4 (10)
MB (2nd marginal obtusis branch), n (%)	8 (6)	7 (8)	1 (3)
MB (intermediate branch), n (%)	2 (2)	1 (1)	1 (3)

segment (p<0.0001) (table 2a). Atherosclerotic plaque in bridged LAD segments was less common than it was in corresponding LAD segments without MBs, with plaque being detected in 2 patients (5%) versus 44 patients (50%), respectively (p<0.0001).

The presence of an MB compared with the absence of an MB in the LAD was associated with a lower calcium score in the bridged segment (OR 0.06, 95% CI 0.01-0.25) (table 2b). This association did not substantially change after adjusting for age, gender, or total coronary calcium score, and after exclusion of patients with an MB with a thickness <3 mm (OR 0.08, 95% CI 0.02-0.37).

The inverse association between MBs and local calcium score was attenuated after adjusting for local perivascular adipose tissue thickness (OR 0.35, 95% CI 0.04-2.70). The mean thickness of perivascular adipose tissue in the region of interest was significantly associated with the local coronary calcium score per millimetre in the 88 subjects without an MB (β adjusted for age and gender (OR 0.21, 95% CI 0.07-0.34).

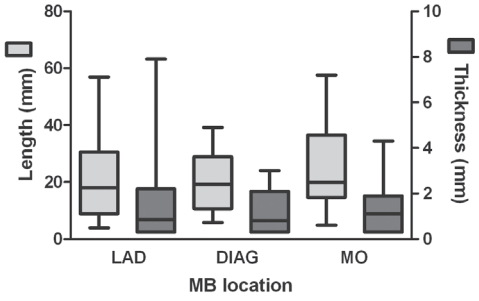

Figure 2 | Length and maximal thickness of myocardial bridges (MBs).
Boxplot depicting MB length (left y-axis) and MB thickness (right y-axis).
LAD: left anterior descending artery; DIAG diagonal branches; MO: marginal obtusis branches of the circumflex coronary artery

Table 2 | Calcium content in segments of the left anterior descending (LAD) coronary artery with a myocardial bridge (MB) and corresponding regions without an MB

A: calcium score in LAD segments with an MB and in LAD segments without an MB

Calcium score:	0.0	0.1-10.0	10.0-120.0
No myocardial bridge of the LAD	46 (52%)	24 (27%)	18 (21%)
Myocardial bridge of the LAD	38 (95%)	1 (3%)	1 (3%)

p<0.0001

B: relationship between presence of an MB and calcium score adjusted for confounding factors

	Model	OR (95% CI)*
Myocardial bridge (yes/no)	1	0.06 (0.01-0.25)
	2	0.05 (0.01-0.22)
	3	0.01 (0.00-0.13)
	4	0.35 (0.04-2.70)

Model 1: crude
Model 2: adjusted for age and gender
Model 3: model II additionally adjusted for total coronary artery calcium score
Model 4: model II additionally adjusted for local perivascular adipose tissue thickness of the LAD
* OR indicates the change in local log-transformed calcium score (0 or >0) in the presence of a myocardial bridge compared to absence of a myocardial bridge. † p< 0.001

Table 3 | Risk factors for atherosclerosis in relation to the presence of a myocardial bridge (MB) at any location

Subjects with MB (n=56) versus subjects without MB (n=72)	OR (95% CI)
Age (per year)	1.00 (0.95-1.07)
Gender (male)	1.60 (0.74-3.47)
Current smoking (yes)	1.33 (0.59-3.01)*
Diabetes (yes)	0.89 (0.36-2.20)*
BMI (per kg/m^2)	0.96 (0.89-1.06)*
LDL-cholesterol (mmol/l)	1.17 (0.83-1.66)*
Triglycerides (mmol/l)	1.24 (0.85-1.83)*
Epicardial adipose tissue volume (per cm^3)	1.00 (0.99-1.01)*
Local perivascular adipose tissue of the LAD(per mm^2)	0.89 (0.75-1.07)*
Metabolic syndrome (yes)	1.02 (0.49-2.09)*
Number of diseased vessels (0,1,2,3)	1.39 (0.93-2.08)*
Calcium score of the LAD (Agatston) (per 100 units)	0.94 (0.77-1.14)*
Total calcium score (Agatston) (per 100 units)	1.01 (0.95-1.08)*

*adjusted for age and gender

Determinants of presence of myocardial bridges at any location

The presence of an MB at any location was not associated with age (per year OR 1.00, 95% CI 0.95-1.07), male gender (OR 1.60, 95% CI 0.74-3.47), or other risk factors for cardiovascular disease, such as smoking, diabetes, BMI, LDL-cholesterol, triglycerides, epicardial adipose tissue volume, and presence of the metabolic syndrome (table 3). Moreover, the extent of coronary artery disease, expressed as the number of diseased vessels, total calcium score, and calcium score of the LAD, was also not associated with the presence of an MB.

DISCUSSION

In this cross-sectional study of patients referred for investigation of stable or unstable angina pectoris, coronary artery segments covered with an MB had a lower calcium score compared with segments without an MB. The characteristics of patients with and without an MB of the LAD were comparable. The inverse association between MBs and local calcium scores was independent of age, gender, and total coronary artery calcium score, but was influenced by the thickness of local perivascular adipose tissue.

The absence of atherosclerosis in coronary artery segments covered by an MB has been reported in post-mortem and imaging studies[1;3;5-9;24]. By comparing bridged LAD segments to matched control segments, we could confirm that the absence of atherosclerosis is due to the MB and is not a random observation. This is in line with earlier findings of studies investigating the severity of atherosclerosis relative to the distance from the ostium in groups with and without MBs[5;24]. Moreover, although this study was only hypothesis generating, we demonstrated that perivascular adipose tissue has a role in the association between MBs and atherosclerosis.

In a post-mortem study it was shown that the intima of bridged LAD segments is thinner, indicating less atherosclerotic transformation, than that of comparable segments of coronary artery without an MB[4]. Furthermore, the extent of atherosclerosis in each subsequent LAD cross-section is less in LAD segments with an MB than in segments without an MB[5;24]. CT studies have shown that bridged segments are less affected by, or are even free of, atherosclerosis[7;9;20], although corresponding segments without MB were not evaluated. An ultrasound study revealed atherosclerotic plaque in 9.5% of bridged coronary artery segments compared with 81.5% of corresponding segments without an MB[25].

The mechanism by which bridged coronary artery segments are protected against atherosclerosis remains to be elucidated. That the association between MBs and local calcium scores changed after adjusting for local perivascular adipose tissue suggests that perivascular adipose tissue influences this association. There is more perivascular adipose tissue at sites of atherosclerosis[26], and this tissue can secrete pro-inflammatory adipokines and cytokines[27]. In vitro, adipose tissue-conditioned medium induces the adhesion of monocytes to endothelial cells and the migration of monocytes[11;12]. In mice, perivascular adipose tissue has been found to promote the migration of macrophages towards the vascular wall[28]. As perivascular adipose tissue does not come into contact with the vascular wall if there is an MB, the latter may protect the vascular wall against pro-inflammatory cytokines and adipokines secreted by perivascular adipose tissue and thus inhibit the atherosclerotic process.

An increase in shear stress at the site of an MB has been proposed as explanation for the decrease in atherosclerosis[10;29]. Endothelial cells beneath an MB are spindle shaped, indicating that they are subjected to high shear stress, whereas endothelial cells in other regions show a polygonal distribution, indicating that they are subjected to low shear stress[10]. An increase in shear stress is thought to hinder monocyte adhesion and lipid transfer across the vascular wall. An argument against the role of hemodynamic forces is that endothelial cell function is impaired at the site of an MB[25], and endothelial dysfunction is associated with a high intravascular pressure. Enhanced lymphatic drainage caused by compression during systole has recently been proposed as an alternative explanation for the reduced atherosclerotic burden in bridged segments[30]. Moreover, coronary artery segments proximal to an MB are affected more by atherosclerosis than are proximal segments in subjects without MB[5]. Intravascular pressure is higher in segments proximal to an MB than in the bridged segment itself[31], and increased pressure on the vascular wall and non-laminar flow give rise to advanced plaque[5;24;32]. The prevalence of MBs is high in subjects with a history of myocardial infarction (46%), and MB thickness is greater in these subjects than in subjects without a history of myocardial infarction[5]. It can be concluded that MB thickness is associated with the extent of atherosclerosis in the coronary artery segment proximal to the MB. Owing to the low occurrence of calcified plaque in bridged segments, we were not able to evaluate the association between MB thickness and atherosclerosis in the present study.

The prevalence of MBs (44%) was high in the present study. The broad range in prevalence reported in the literature (3.5-86%) may be due to the use of different detection methods, such as autopsy or angiography, and to the use of different definitions of MBs[2;2;33]. In CT studies, MBs were detected in 3.5-55% of the study population[33;34] compared with 44% in our study. Another explanation is that thin MBs might be taken into account in studies with a high prevalence but not in studies with a low prevalence.

In this study with subjects referred for angina pectoris, we confirmed that presence of an MB is not influenced by age, gender or risk factors for cardiovascular disease. Also, the point estimate of the

relation between presence of an MB and calcium-score did not change after adjustment for age and gender. This implicates that MBs influence the atherosclerotic process independent from age and gender.

We acknowledge study limitations. Due to the cross-sectional design it is not possible to make inferences about causality. Also, we used calcium score in coronary segments as a surrogate measure of atherosclerosis. Although calcium deposition is highly correlated with atherosclerotic plaque burden[35], the calcium content is heterogeneous in different plaques[36]. Calcium score has a high sensitivity, but a moderate specificity for predicting the presence of a significant stenosis[35]. Outward remodelling reduces the association between atherosclerosis and vascular stenosis[37]. Moreover, an increased calcium content of an atherosclerotic plaque is a sign of atherosclerosis of the vascular wall and not of plaque rupture. For this reason, we also investigated atherosclerosis by determining the presence of atherosclerotic plaque. Information on the presence of plaque confirmed the results obtained using calcium score. Intravascular ultrasound has been used to detect MBs and to determine plaque composition[31], and this imaging method could be used to further explore the role of perivascular adipose tissue.

In conclusion, in patients with stable and instable angina pectoris, coronary artery segments covered with an MB have a lower Agatston calcium score than segments without an MB. The association between MB and calcium score was influenced by the thickness of perivascular adipose tissue adjacent to the coronary artery segment.

Chapter 6

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REFERENCE LIST

1 Burnsides C, Edwards JC, Lansing AI, Swarm RL. Arteriosclerosis in the intramural and extramural portions of coronary arteries in the human heart. Circulation 1956 Feb;13(2):235-41.

2 Mohlenkamp S, Hort W, Ge J, Erbel R. Update on myocardial bridging. Circulation 2002 Nov 12;106(20):2616-22.

3 Polacek P, Kalove H. Relation of myocardial bridges and loops on the coronary arteries to coronary occulsions. Am Heart J 1961 Jan;61:44-52.

4 Geiringer E. The mural coronary. Am Heart J 1951 Mar;41(3):359-68.

5 Ishikawa Y, Akasaka Y, Suzuki K, et al. Anatomic properties of myocardial bridge predisposing to myocardial infarction. Circulation 2009 Aug 4;120(5):376-83.

6 Jodocy D, Aglan I, Friedrich G, et al. Left anterior descending coronary artery myocardial bridging by multislice computed tomography: correlation with clinical findings. Eur J Radiol 2010 Jan;73(1):89-95.

7 Kawawa Y, Ishikawa Y, Gomi T, et al. Detection of myocardial bridge and evaluation of its anatomical properties by coronary multislice spiral computed tomography. Eur J Radiol 2007 Jan;61(1):130-8.

8 Lubarsky L, Gupta MP, Hecht HS. Evaluation of myocardial bridging of the left anterior descending coronary artery by 64-slice multidetector computed tomographic angiography. Am J Cardiol 2007 Oct 1;100(7):1081-2.

9 Zeina AR, Odeh M, Blinder J, Rosenschein U, Barmeir E. Myocardial bridge: evaluation on MDCT. AJR Am J Roentgenol 2007 Apr;188(4):1069-73.

10 Ishii T, Asuwa N, Masuda S, Ishikawa Y. The effects of a myocardial bridge on coronary atherosclerosis and ischaemia. J Pathol 1998 May;185(1):4-9.

11 Henrichot E, Juge-Aubry CE, Pernin A, et al. Production of chemokines by perivascular adipose tissue: a role in the pathogenesis of atherosclerosis? Arterioscler Thromb Vasc Biol 2005 Dec;25(12):2594-9.

12 Karastergiou K, Evans I, Ogston N, et al. Epicardial adipokines in obesity and coronary artery disease induce atherogenic changes in monocytes and endothelial cells. Arterioscler Thromb Vasc Biol 2010 Jul;30(7):1340-6.

13 Verhagen SN, Visseren FL. Perivascular adipose tissue as a cause of atherosclerosis. Atherosclerosis 2011 Jan;214(1):3-10.

14 Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. Nature 2006 Dec 14;444(7121):875-80.

15 Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 2008 Dec;29(24):2959-71.

16 Gorter PM, van Lindert AS, de Vos AM, et al. Quantification of epicardial and peri-coronary fat using cardiac computed tomography; reproducibility and relation with obesity and metabolic syndrome in patients suspected of coronary artery disease. Atherosclerosis 2008 Apr;197(2):896-903.

17 Grundy SM, Cleeman JI, Daniels SR, et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 2005 Oct 25;112(17):2735-52.

18 Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 1998 Jul;15(7):539-53.

19 Scanlon PJ, Faxon DP, Audet AM, et al. ACC/AHA guidelines for coronary angiography. A report of the American College of Cardiology/American Heart Association Task Force on practice guidelines (Committee on Coronary Angiography). Developed in collaboration with the Society for Cardiac Angiography and Interventions. J Am Coll Cardiol 1999 May;33(6):1756-824.

20 Canyigit M, Hazirolan T, Karcaaltincaba M, et al. Myocardial bridging as evaluated by 16 row MDCT. Eur J Radiol 2009 Jan;69(1):156-64.

21 Rutten A, Isgum I, Prokop M. Coronary calcification: effect of small variation of scan starting position on Agatston, volume, and mass scores. Radiology 2008 Jan;246(1):90-8.

22 Agatston AS, Janowitz WR, Hildner FJ, et al. Quantification of coronary artery calcium using ultrafast computed tomography. J Am Coll Cardiol 1990 Mar 15;15(4):827-32.

23 Coats AJ. Ethical authorship and publishing. Int J Cardiol 2009 Jan 9;131(2):149-50.

24 Ishikawa Y, Akasaka Y, Ito K, et al. Significance of anatomical properties of myocardial bridge on atherosclerosis evolution in the left anterior descending coronary artery. Atherosclerosis 2006 Jun;186(2):380-9.

25 Kim JW, Seo HS, Na JO, et al. Myocardial bridging is related to endothelial dysfunction but not to plaque as assessed by intracoronary ultrasound. Heart 2008 Jun;94(6):765-9.

26 Mahabadi AA, Reinsch N, Lehmann N, et al. Association of pericoronary fat volume with atherosclerotic plaque burden in the underlying coronary artery: a segment analysis. Atherosclerosis 2010 Jul;211(1):195-9.

27 Mazurek T, Zhang L, Zalewski A, et al. Human epicardial adipose tissue is a source of inflammatory mediators. Circulation 2003 Nov 18;108(20):2460-6.

28 Police SB, Thatcher SE, Charnigo R, Daugherty A, Cassis LA. Obesity promotes inflammation in periaortic adipose tissue and angiotensin II-induced abdominal aortic aneurysm formation. Arterioscler Thromb Vasc Biol 2009 Oct;29(10):1458-64.

29 Scher AM. Absence of atherosclerosis in human intramyocardial coronary arteries: a neglected phenomenon. Atherosclerosis 2000 Mar;149(1):1-3.

30 Erbel R, Ge J, Mohlenkamp S. Myocardial bridging: a congenital variant as an anatomic risk factor for myocardial infarction? Circulation 2009 Aug 4;120(5):357-9.

31 Ge J, Erbel R, Gorge G, Haude M, Meyer J. High wall shear stress proximal to myocardial bridging and atherosclerosis: intracoronary ultrasound and pressure measurements. Br Heart J 1995 May;73(5):462-5.

32 Shinjo SK, Prates NE, Oba SM, Sampaio LO, Nader HB. Distribution and composition of glycosaminoglycans in the left human coronary arterial branches under myocardial bridge. Atherosclerosis 1999 Apr;143(2):363-8.

33 Kantarci M, Duran C, Durur I, et al. Detection of myocardial bridging with ECG-gated MDCT and multiplanar reconstruction. AJR Am J Roentgenol 2006 Jun;186(6 Suppl 2):S391-S394.

34 Wang MH, Sun AJ, Qian JY, et al. Myocardial bridging detection by non-invasive multislice spiral computed tomography: comparison with intravascular ultrasound. Chin Med J (Engl) 2008 Jan 5;121(1):17-21.

35 Haberl R, Becker A, Leber A, et al. Correlation of coronary calcification and angiographically documented stenoses in patients with suspected coronary artery disease: results of 1,764 patients. J Am Coll Cardiol 2001 Feb;37(2):451-7.

36 Moselewski F, O'Donnell CJ, Achenbach S, et al. Calcium concentration of individual coronary calcified plaques as measured by multidetector row computed tomography. Circulation 2005 Jun 21;111(24):3236-41.

37 Erbel R, Mohlenkamp S, Kerkhoff G, Budde T, Schmermund A. Non-invasive screening for coronary artery disease: calcium scoring. Heart 2007 Dec;93(12):1620-9.

Part II

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Chapter 7

Insulin resistance increases the occurrence of new cardiovascular events in patients with manifest arterial disease without known diabetes. The SMART study.

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ABSTRACT

Background: Insulin resistance is accompanied by a cluster of metabolic changes, often referred to as metabolic syndrome. Metabolic syndrome is associated with an increased cardiovascular risk in patients with manifest arterial disease. We investigated whether insulin resistance is associated with an increased risk for cardiovascular events in patients with manifest arterial disease without known diabetes and whether this can be explained by the components of the metabolic syndrome or by inflammation.

Methods: Prospective cohort study in 2611 patients with manifest arterial disease without known diabetes. Homeostasis model of insulin resistance (HOMA-IR) was used to quantify insulin resistance. The relation of HOMA-IR with cardiovascular events (vascular death, myocardial infarction or stroke) and all cause mortality was assessed with Cox regression analysis. In additional models adjustments were performed for the single components constituting the metabolic syndrome and for inflammation.

Results: HOMA-IR increases with the number of metabolic syndrome components (mean HOMA-IR ± SD in groups with 0, 1, 2, 3, 4 and 5 metabolic syndrome components: 1.4 ± 0.7; 1.8 ± 1.2; 2.4 ± 1.5; 3.1 ± 1.8; 4.0 ± 2.6; and 5.6 ± 3.6 respectively). High HOMA-IR was independently associated with an increased risk of cardiovascular events (tertile 2 vs. 1 HR 1.92; 95%CI 1.20-3.08) (tertile 3 vs.1 HR 1.78; 95%CI 1.10-2.89) and with all cause mortality (tertile 2 vs. 1 HR 1.80; 95%CI 1.04-3.10) (tertile 3 vs.1 HR 1.56; 95%CI 0.88-2.75). These relations were not influenced by the individual components of metabolic syndrome or by inflammation.

Conclusions: In patients with manifest arterial disease without known diabetes, insulin resistance increases with the number of metabolic syndrome components, and elevated insulin resistance increases the risk of new cardiovascular events.

BACKGROUND

Both insulin resistance and metabolic syndrome are recognized as important factors in the development of cardiovascular disease[1,2]. Obesity-induced insulin resistance is considered to be the major driver of the clustering of interrelated metabolic disturbances (e.g. dyslipidemia, hyperglycemia, elevated blood pressure)[3], often referred to as metabolic syndrome[4], thereby leading to an increased cardiovascular risk. Although insulin resistance may be the unifying pathophysiological mechanism underlying the metabolic syndrome[5], there is uncertainty regarding the independent role of insulin resistance in the development of atherosclerotic vascular disease[1,5-12], also in patients with arterial diseases[13,14].

Metabolic syndrome is highly prevalent in patients with manifest arterial disease (46%)[15] and is associated with advanced vascular damage[16], thereby identifying those patients with an even higher cardiovascular risk. This high cardiovascular risk may be due to a combination of non-classical risk factors associated with insulin resistance, e.g. inflammation, hyperinsulinemia, oxidative stress, and hypercoagulability, together with the separate components of metabolic syndrome (low high-density lipoprotein (HDL)-cholesterol, elevated triglycerides, elevated glucose, elevated blood pressure, increased waist circumference)[17-19]. Nevertheless, the magnitude of the association between insulin resistance and the occurrence of new vascular events in these high-risk patients is not yet elucidated. In addition, it is not known whether insulin resistance *per se* has an influence on the elevated cardiovascular risk or that it is mediated by the components of metabolic syndrome.

Aim of the current study is (1) to investigate the relation between insulin resistance and metabolic disturbances, (2) to determine whether insulin resistance, derived by homeostasis model assessment of insulin resistance (HOMA-IR), is associated with increased occurrence of cardiovascular events and all cause mortality, and (3) to evaluate to what extent this relation can be explained by the individual components of the metabolic syndrome or by inflammation.

METHODS

Study settings, participants and design

In this study, we used data from patients enrolled in the Second Manifestations of Arterial disease (SMART) study. The SMART study is an ongoing prospective single-centre cohort study in patients with manifest arterial disease or cardiovascular risk factors.[20] Started in September 1996, patients aged 18-80, were referred to the University Medical Centre (UMC) Utrecht with a recent diagnosis of manifest arterial disease or a cardiovascular risk factor. Not approached are patients with terminal malignant disease, those not independent in daily activities (Rankin scale >3) or not sufficiently fluent in Dutch. Patients entered the SMART study if participation in the study was supported by the treating specialist and if the patients themselves consented to participate. The Medical Ethics Committee approved the study, and all participants gave their written informed consent. The rationale and design of the SMART study and a detailed description of the criteria used to define the different manifest arterial diseases were published previously[20].

For the current study, the data of the 3239 participants with clinical manifestations of arterial disease (coronary heart disease, cerebrovascular disease, peripheral arterial disease or abdominal aortic

aneurysm) included between July 1, 2003 and March 1, 2010 were considered. A total of 548 patients with known Type 1 or Type 2 diabetes mellitus (T2DM) were not included in the analyses. Diabetes mellitus at baseline was defined as a referral diagnosis of diabetes, self-reported diabetes, or use of glucose lowering agents. If glucose was ≥7 mmol/l without a history of diabetes, patients were only included if they were not on glucose lowering therapy after 1 year follow up[21]. In addition, patients with missing data on glucose or insulin were excluded (n=44).

Data acquisition

All baseline measurements were performed on a single day at the UMC Utrecht. Medical history, use of current medication and current and past cigarette smoking behavior were derived from a questionnaire described elsewhere[20]. Height, weight, waist circumference and blood pressure were measured. Blood samples were collected after an overnight fast. Total cholesterol, triglycerides, HDL-cholesterol, creatinin, and high sensitive-C-reactive protein (hs-CRP) levels were measured. Hs-CRP measurements below the lower limit of detection of 0.2 mg/l (n=45) were set at 0.2 mg/l. Plasma glucose was measured using commercial enzymatic dry chemistry kits (Johnson and Johnson). Plasma insulin was measured with an immunometric technique on an IMMULITE 1000 Analyzer (Diagnostic Products Corporation, Los Angeles, USA). Inter-assay coefficient of variation for insulin measurements was 9% at 7 mIU/l and <5.5% at 20-120 mIU/l. Insulin measurements below the lower limit of detection of 2 mIU/l (n= 74) were set at 2 mIU/l.

HOMA-IR was used as quantitative estimate of the degree of insulin resistance at baseline. The value for insulin resistance can be assessed by the formula: HOMA-IR = (fasting serum glucose (mmol/l) x fasting serum insulin (mIU/l) / 22.5)[22]. HOMA-IR correlates well with measurements obtained by means of the euglycemic clamp technique[23]. Therefore it provides a reliable approach to estimate insulin resistance and lends itself for use in large epidemiological studies[24]. In addition, quantitative insulin sensitivity check index (QUICKI) was measured to compare the results retrieved with HOMA. QUICKI was calculated with the formula QUICKI= 1/(log(insulin x glucose))[25].

Metabolic syndrome was defined according to the Adult Treatment Panel (ATP) III criteria[4]. Metabolic syndrome requires the presence of at least three of the following metabolic abnormalities: abdominal obesity (waist circumference >102 cm in men and >88 cm in women), high blood pressure (≥130 mmHg systolic or ≥85 mmHg diastolic), hypertriglyceridemia (serum triglycerides ≥1.70 mmol/l (150 mg/dl)), low HDL-cholesterol (serum HDL-cholesterol <1.04 mmol/l (40 mg/dl) in men and <1.29 mmol/l (50 mg/dl) in women), high fasting glucose (fasting serum glucose ≥5.6 mmol/l (100 mg/dl)). Patients on blood pressure-lowering medication were regarded as having high blood pressure.

Follow up

All patients were asked to complete a questionnaire every 6 months. The questionnaire comprised of questions on hospital admissions and out-patient clinic visits in the preceding 6 months. If patients reported a possible event, hospital discharge letters and results of relevant laboratory results and radiology examinations were collected and evaluated by an Outcome Event Committee comprising of physicians from different departments. Outcome of interest for the present study was a composite endpoint of fatal or non-fatal cardiovascular events during follow-up. A cardiovascular event was defined as the occurrence of cardiovascular death, ischemic stroke or myocardial infarction (table 1). Forty-two out of 2611 patients (2%) were lost to follow-up.

Duration of follow-up was defined as the period between the date of study inclusion and the date of the occurrence of a new cardiovascular event, date of loss to follow-up or the pre-selected closing date of March 1st, 2011.

Data analysis

Baseline characteristics were reported across tertiles of HOMA-IR. Mean and standard deviation (SD) were reported if continuous variables were normally distributed and median and interquartile range if distributions were skewed. Differences between tertiles of HOMA-IR were tested with one-way ANOVA (continuous normal distributed variables) or Kruskal-Wallis test (continuous skewed variables).
A trend between the number of components of metabolic syndrome and HOMA-IR was investigated with ANCOVA (general linear model procedure), and adjusted for age and gender.
The hazard ratios (HR) with corresponding 95% confidence intervals (CI) for the occurrence of a new cardiovascular event and of total mortality associated with insulin resistance were estimated with Cox proportional hazards analysis. Three models were used to estimate the relation between HOMA-IR as independent variable and new cardiovascular events or total mortality as dependent variables. Model 1 was adjusted for age and gender. In model 2 additional adjustments were made for current smoking, use of lipid lowering medication and blood pressure lowering medication. In model 3 exploratory analyses are shown with additional adjustments for history of coronary artery disease, cerebrovascular disease, abdominal aortic aneurysm and peripheral arterial disease on top of model 2. Analyses were repeated with QUICKI as independent variable.

Table 1 | Definition of study outcome events

Stroke	Definite: relevant clinical features causing an increase in impairment of at least one grade on the modified Rankin scale, accompanied by an infarction on repeat brain imaging. Probable: clinical features that have caused an increase in impairment of at least one grade on the modified Rankin scale; without documentation by means of brain imaging.
Myocardial infarction	At least two of the following criteria: (I) chest pain for at least 20 min, not disappearing after administration of nitrates; (II) ST-elevation > 1 mm in two following leads or a left bundle branch block on the electrocardiogram; (III) Creatinine kinase (CK) elevation of at least two times the normal value of CK and a myocardial band-fraction > 5% of the total CK.
Vascular mortality	Death from ischemic or hemorrhagic stroke, congestive heart failure, myocardial infarction or rupture of abdominal aortic aneurysm. Sudden death: unexpected cardiac death occurring within 1 hour after onset of symptoms, or within 24 hours given convincing circumstantial evidence. Vascular death from other causes
Composite vascular outcome event	A composite of stroke, myocardial infarction and vascular mortality completed with a probable or definite retinal infarction or bleeding and probable or definite hemorrhagic stroke.
All-cause mortality	Death from any cause

To investigate whether the relation between insulin resistance and cardiovascular disease can be explained by the single components of the metabolic syndrome or by inflammation, additional adjustments for waist circumference, HDL-cholesterol, triglycerides, fasting glucose, systolic blood pressure and hs-CRP were performed on top of model 2 (model 4). Significance was taken at the 5% level (two-sided).

To investigate whether the relation between HOMA-IR and new cardiovascular events is modified by other variables, interaction terms for age, gender, waist circumference and smoking were subsequently included in model 2. The p-values of these interaction terms were >0.05 and it was concluded that no effect modification was present for these variables.

In the Cox regression analyses, single imputation methods were used to reduce missing covariate data for smoking (n=3 (<1%)), HDL-cholesterol (n=4 (<1%)), triglycerides (n=2 (<1%)), systolic blood pressure (n=13 (<1%)), waist circumference (n=76 (3%)), hs-CRP (n=11 (<1%)), since incomplete case analysis leads to loss of statistical power and possibly bias.

RESULTS

Study population

The mean age was 59.6 ± 10.4 years and 71% of patients were male. HOMA-IR levels were 2.9 ± 2.2 for males and 2.5 ± 1.8 for females. CIMT and incidence of albuminuria did not change substantially across tertiles of HOMA-IR. Further general characteristics of the study population according to tertiles of HOMA-IR are listed in table 2.

Insulin resistance and metabolic disturbances

Prevalence of metabolic syndrome was 42%. Metabolic syndrome was more prevalent in the highest HOMA-IR tertile compared with the lowest tertile (69% versus 16%) (table 2). Waist circumference, fasting glucose level, triglyceride level and systolic blood pressure were higher in patients within the highest HOMA-IR tertile, whereas HDL-cholesterol level was lower. Level of hs-CRP was incurred with increasing levels of insulin resistance. It is shown that HOMA-IR increases with an increment in the number of components of metabolic syndrome, adjusted for age and gender (p-value for trend <0.001). (table 3)

Insulin resistance and occurrence of cardiovascular events

During a mean follow-up of 3.1 ± 1.9 years (total number of follow-up years 8094), 91 out of 2611 subjects died (of whom 47 due to a cardiovascular cause), 74 experienced a myocardial infarction and 34 an ischemic stroke. This corresponds with a cardiovascular event rate of 16 per 1000 patient years and a mortality rate of 11 per 1000 patient years. HOMA-IR was associated with an increased risk for the occurrence of a new vascular event, adjusted for age, gender, smoking and use of lipid lowering and blood pressure lowering medication (tertile 2 versus 1 HR 1.92 (95% confidence interval (CI) 1.20-3.08); (tertile 3 versus 1 HR1.78 (95%CI 1.10-2.89) (table 4)). Comparison of the lower tertiles of QUICKI with the highest tertile yielded exactly the same HRs as comparison of the higher HOMA-IR tertiles with the first tertile (table 5). Risk of death from any cause was increased in the second tertile of HOMA-IR versus the first (HR 1.80, (95%CI 1.05-3.10) and this risk was also increased, although non-statistically significant, in the third versus the first tertile (HR 1.56 (95%CI: 0.88-2.75).

Table 2 | Baseline characteristics of the study population (n=2611) according to tertiles of HOMA-IR

HOMA-IR, tertiles	**Tertile 1**	**Tertile 2**	**Tertile 3**
HOMA-IR	1.1±0.4	2.3±0.4	4.9±2.3
(Range)	0.4-1.7	1.7-3.0	3.0-23.3
	n=872	n=870	n=869
Age (years)	59.4 ± 10.5	60.0 ± 10.4	59.4 ± 10.4
Male gender, n (%)	593 (68)	611 (70)	658 (76)
Body mass index (kg/m^2)	24.7 ± 3.1	26.7 ± 3.4	28.9 ± 4.2
Abdominal adipose tissue (cm)	7.6 ± 2.0	8.7 ± 2.2	10.1 ± 2.5
Current smoking, n (%)	288 (33)	245 (28)	251 (29)
Creatinin clearance MDRD (ml/min/1.73m^2)	79.0 ± 16.8	75.4 ± 16.4	74.0 ± 18.3
Medication			
Lipid-lowering agents, n (%)	624 (72)	671 (77)	709 (82)
Anti-platelet agents, n (%)	689 (79)	723 (83)	724 (83)
Blood pressure-lowering agents, n (%)	588 (67)	654 (75)	751 (86)
Location of manifest arterial disease			
Cerebrovascular disease, n (%)	263 (30)	261 (30)	206 (24)
Coronary heart disease, n (%)	509 (58)	540 (62)	614 (71)
Peripheral arterial disease, n (%)	138 (16)	127 (15)	133 (16)
Abdominal aortic aneurysm, n (%)	56 (6)	64 (7)	44 (5)
Atherosclerotic burden			
CIMT(mm)	0.88 ± 0.24	0.89 ± 0.25	0.89 ±0.22
Albumin/creatinin ratio >3.0 mg/mmol, n (%)	120 (14)	120 (14)	135 (16)
Metabolic syndrome components			
Metabolic syndrome (ATPIII), n (%)	148 (17)	232 (37)	582 (67)
Waist circumference (cm)	87.6 ± 10.6	94.0 ±10.5	100.5 ± 11.8
Blood pressure systolic (mmHg)	138 ± 20	141 ± 21	142 ± 21
Blood pressure diastolic (mmHg)	81 ±11	83 ± 11	84 ± 11
Fasting glucose (mmol/l)	5.4 ± 0.5	5.7 ± 0.6	6.1 ± 0.8
Triglycerides (mmol/l)	1.0 (0.8-1.4)	1.2 (0.9-1.7)	1.1 (0.9-1.3)
HDL-cholesterol (mmol/l)	1.4 ± 0.4	1.3 ± 0.4	1.1 ± 0.3
LDL-cholesterol (mmol/l)	2.7 ± 0.9	2.7 ± 1.0	2.6 ± 0.9
hs-CRP (mg/l)	1.4 (0.6-3.2)	1.6 (0.8-3.7)	2.1 (1.0-4.3)
Fasting insulin (mIU/l)	4.8 ± 1.6	9.1 ± 1.5	17.9 ± 7.8
QUICKI	0.32 ± 0.05	0.26 ± 0.01	0.22 ± 0.02

Table 3 | HOMA-IR in relation to metabolic syndrome (ATPIII) and the number of metabolic syndrome components in patients with manifest arterial disease.

n= 2611		N	HOMA-IR	QUICKI	p-value
Metabolic syndrome	no	1478	2.1 ± 1.4	0.28±0.05	<0.001
	yes	1053	3.7 ± 2.5	0.24±0.04	
Metabolic syndrome components	0	137	1.4 ± 0.7	0.31±0.05	<0.001
	1	547	1.8 ± 1.2	0.30±0.06	
	2	794	2.4 ± 1.5	0.27±0.05	
	3	573	3.1 ± 1.8	0.25±0.04	
	4	366	4.0 ± 2.6	0.23±0.03	
	5	114	5.6 ± 3.6	0.22±0.03	

Table 4 | HOMA-IR and the risk for new cardiovascular events (vascular death, myocardial infarction or stroke) in patients with manifest arterial disease (n=2611).

HOMA-IR		tertile 1		tertile 2		tertile 3	
	Model	# events	HR (95%CI)	# events	HR (95%CI)	# events	HR (95%CI)
All vascular events	1	26	1 (reference)	52	1.86 (1.16-2.98)	48	1.70 (1.05-2.74)
	2		1 (reference)		1.92 (1.20-3.08)		1.78 (1.10-2.89)
	3		1 (reference)		1.86 (1.16-3.00)		1.79 (1.10-2.90)
	4		1 (reference)		1.80 (1.10-2.96)		1.50 (0.85-2.68)
Myocardial infarction	1	17	1 (reference)	30	1.63 (0.90-2.96)	27	1.42 (0.77-2.61)
	2		1 (reference)		1.61 (0.89-2.93)		1.41 (0.76-2.61)
	3		1 (reference)		1.57 (0.86-2.85)		1.39 (0.75-2.59)
	4		1 (reference)		1.34 (0.72-2.51)		0.95 (0.46-1.98)
All cause mortality	1	20	1 (reference)	39	1.69 (0.99-2.91)	32	1.43 (0.82-2.51)
	2		1 (reference)		1.80 (1.05-3.10)		1.56 (0.88-2.75)
	3		1 (reference)		1.76 (1.02-3.03)		1.63 (0.92-2.88)
	4		1 (reference)		1.83 (1.04-3.24)		1.56 (0.78-3.09)
Vascular mortality	1	9	1 (reference)	23	2.26 (1.04-4.88)	15	1.50 (0.66-3.43)
	2		1 (reference)		2.42 (1.12-5.25)		1.72 (0.74-3.98)
	3		1 (reference)		2.24 (1.03-4.88)		1.74 (0.75-4.04)
	4		1 (reference)		2.56 (1.15-5.70)		1.85 (0.73-4.73)

Model I: adjusted for age and gender
Model II: model I additionally adjusted for current smoking, lipid lowering medication, blood pressure-lowering medication
Model III: model II additionally adjusted for history of coronary artery disease, cerebrovascular disease, abdominal aortic aneurysm and peripheral arterial disease.
Model IV: model II additionally adjusted for waist circumference, systolic blood pressure, triglycerides, HDL-c, glucose and hs-CRP

Table 5 | QUICKI and the risk for new cardiovascular events (vascular death, myocardial infarction or stroke) in patients with manifest arterial disease (n=2611).

QUICKI		tertile 1		tertile 2		tertile 3	
	Model	# events	HR (95%CI)	# events	HR (95%CI)	# events	HR (95%CI)
All vascular events	1	48	1.70 (1.05-2.74)	52	1.86 (1.16-2.98)	26	1 (reference)
	2		1.78 (1.10-2.89)		1.92 (1.20-3.08)		1 (reference)
	3		1.79 (1.10-2.90)		1.86 (1.16-3.00)		1 (reference)
	4		1.50 (0.85-2.68)		1.80 (1.10-2.96)		1 (reference)
Myocardial infarction	1	27	1.42 (0.77-2.61)	30	1.63 (0.90-2.96)	17	1 (reference)
	2		1.41 (0.76-2.61)		1.61 (0.89-2.93)		1 (reference)
	3		1.39 (0.75-2.59)		1.57 (0.86-2.85)		1 (reference)
	4		0.95 (0.46-1.98)		1.34 (0.72-2.51)		1 (reference)
All cause mortality	1	32	1.43 (0.82-2.51)	39	1.69 (0.99-2.91)	20	1 (reference)
	2		1.56 (0.88-2.75)		1.80 (1.05-3.10)		1 (reference)
	3		1.63 (0.92-2.88)		1.76 (1.02-3.03)		1 (reference)
	4		1.56 (0.78-3.09)		1.83 (1.04-3.24)		1 (reference)
Vascular mortality	1	15	1.50 (0.66-3.43)	23	2.26 (1.04-4.88)	9	1 (reference)
	2		1.72 (0.74-3.98)		2.42 (1.12-5.25)		1 (reference)
	3		1.74 (0.75-4.04)		2.24 (1.03-4.88)		1 (reference)
	4		1.85 (0.73-4.73)		2.56 (1.15-5.70)		1 (reference)

Model I: adjusted for age and gender
Model II: model I additionally adjusted for current smoking, lipid lowering medication, blood pressure-lowering medication
Model III: model II additionally adjusted for history of coronary artery disease, cerebrovascular disease, abdominal aortic aneurysm and peripheral arterial disease.
Model IV: model II additionally adjusted for waist circumference, systolic blood pressure, triglycerides, HDL-c, glucose and hs-CRP

Influence of metabolic syndrome and inflammation on the relation between insulin resistance and cardiovascular risk

To investigate the independent relation between insulin resistance and cardiovascular events, additional analyses were performed adjusting for factors that are possibly in the causal pathway. Adjusting for the individual components of the metabolic syndrome did not substantially change the relation between HOMA-IR and the composite endpoint of all vascular events (tertile 2 versus 1 HR 1.76; 95%CI 1.08-2.89; tertile 3 versus 1 HR 1.48; 95%CI 0.83-2.63). Congruently adjustment for presence of the metabolic syndrome did not alter the point estimate for the relation between HOMA-IR and the composite endpoint (tertile 2 vs 1 HR 1.92 95%CI 1.20-3.08; tertile 3 vs1 (HR 1.78; 95%CI 1.10-2.90). Moreover, the relationship was not altered after adjusting for hs-CRP as a marker of inflammation (tertile 2 versus 1 HR 1.93; 95%CI 1.20-3.10; tertile 3 versus 1 HR 1.73; 95%CI 1.07-2.82). Hazard ratios for analyses with the single components of the metabolic syndrome and hs-CRP are depicted in table 4, model 4.

DISCUSSION

In patients with manifest arterial diseases without known diabetes, insulin resistance was elevated in patients with a higher number of metabolic syndrome components pointing towards a causal role of insulin resistance in the pathophysiology of metabolic syndrome. Furthermore, in this population, insulin resistance is associated with an increased risk to develop a second vascular event, independent of risk factors clustering in the metabolic syndrome including inflammation. Insulin resistance also tended to be associated with an increased risk of death form any cause, although the relationship between insulin resistance and all-cause mortality was not statistically significant in the highest tertile of HOMA-IR

These results imply that insulin resistance is a major driver underlying the clustering of metabolic abnormalities and that insulin resistance is also directly and independently related to an increased risk for cardiovascular events in patients with manifest arterial disease. In general, patients with insulin resistance are at increased cardiovascular risk[1,5-12]. Two other studies have shown that insulin resistance is associated with an increased risk for new vascular events in patients with manifest arterial disease[13,14]. Furthermore HOMA-IR has been found to independently predict the occurrence of a secondary event in patients with acute coronary syndrome[26]. In these studies, HOMA-IR was lower and the range was less broad as compared to the present study. The reason for the relatively high HOMA-IR in our cohort is not fully clear and not attributable to high BMI, since BMI in our study population was comparable or even lower than in these previous studies.

Obesity is associated with the metabolic abnormalities and an increased blood pressure clustered in the metabolic syndrome [3]. With an increase in adipocyte size, capillary density in adipose tissue decreases by an increase in collagen IV in the capillary walls, preventing angiogenesis in vitro[27]. Oxidative stress caused by a decreased capillary density may be the underlying mechanism of adipose tissue dysfunction characterized by secretion of pro-inflammatory cytokines and metabolic changes[28]. As a result of adipose tissue dysfunction efflux of free fatty acids increases, eventually giving rise to systemic dyslipidemia [29] One of the potential mechanisms for obesity-associated hypertension linking hypertension and insulin resistance is a decrease in cardiac natriuretic peptides as observed in insulin resistance [30].

Results of the present study are clinically relevant, since improving insulin sensitivity may improve cardiovascular outcome, as was already suggested in patients with type 2 diabetes [31,32]. In a randomized trial investigating the optimal treatment in diabetic patients with stable ischemic heart disease, there was a trend towards a lower cardiovascular risk for insulin sensitization compared to insulin provision in the group of patients randomized to undergo revascularization as opposed to medical therapy alone[32]. Improving insulin sensitivity with the peroxisome proliferator-activated receptor (PPAR-) γ agonist pioglitazone was demonstrated to reduce the occurrence of macrovascular events in patients with type 2 diabetes[31].

The increased occurrence of cardiovascular events in patients with insulin resistance is most likely due to atherosclerotic disease. Arterial stiffness is considered to be an early manifestation of atherosclerosis. In non-diabetic subjects, fasting glucose was associated with arterial stiffness as measured with pulse wave velocity[33]. In patients without diabetes, pulse wave velocity was an independent predictor for intima media thickness, but not in patients with diabetes[34]. The mechanisms by which insulin resistance leads to atherosclerotic cardiovascular disease are not fully understood. Indirect effects through accelerating chronic inflammation, oxidative stress, and metabolic

abnormalities (e.g. hyperglycemia, dyslipidemia, and elevated blood pressure) have been implicated to contribute to endothelial dysfunction, thereby enhancing atherosclerotic cardiovascular disease[17,35]. In patients with manifest arterial disease the single components of the metabolic syndrome did not increase the risk of new vascular events in contrast to these components combined in the metabolic syndrome[36]. The metabolic syndrome is under debate because of its limited additive value in prediction of cardiovascular disease on top of classical risk factors [37]. However, from an etiologic point of view, the concept of the metabolic syndrome helps us to understand the pathophysiology of insulin resistance and its related metabolic changes leading to an increased cardiovascular risk. In the present study we have evaluated the etiologic relation between insulin resistance and cardiovascular risk. Metabolic syndrome is highly related to insulin resistance.

In our analyses, adjusting for the individual components of the metabolic syndrome did not alter the relation between insulin resistance and the risk of new vascular events. This implies that, besides the components constituting the metabolic syndrome, there are other factors that drive the relationship between insulin resistance and the risk of new cardiovascular endpoints. In adipose tissue dysfunction adipocytes enlarge giving rise to insulin resistance and an increase in adipocytokine production leading to a pro-inflammatory state. However, our analyses do not point to inflammation as a key factor in the relationship between insulin resistance and vascular disease as adjustment for hs-CRP did not substantially influence the point estimate for the risk of cardiovascular events across tertiles of HOMA-IR. Besides pro-inflammatory effects, adipocytokines are known to induce pro-haemostatic, pro-coagulant effects[38], although platelet aggregation is reduced in hyperinsulinemic states[39]. Furthermore endothelial function is impaired in insulin resistance through imbalance of nitric oxide production and the release of vasoconstrictor endothelin-1[40]. Various adipocytokines may also directly affect endothelial function and accelerate processes involved in atherogenesis. Another explanation may be the change in lipoprotein particle composition. Insulin resistance is associated with the pro-atherogenic small dense LDL-cholesterol concentration[41].

We acknowledge some limitations of our study. Compared to other studies with comparable patient groups, there was a relatively low mortality rate, limiting the statistical power of the study. HOMA-IR was used to estimate the level of insulin resistance. Although the hyperinsulinemic euglycemic clamp technique is the standard for measuring insulin resistance[42], this technique is unsuitable for epidemiologic studies. HOMA-IR seems to be a reliable tool in the assessment of insulin resistance due to its strong relation to clamp-measured insulin resistance in both patients with and without diabetes[23,24]. Moreover, repeating analyses with the QUICKI index as a measure of insulin sensitivity confirmed the results retrieved with HOMA-IR. It can be agued that it is not correct to include glucose in models together with HOMA-IR. However since hyperglycemia is one of the effects of insulin resistance and part of the metabolic syndrome just as dyslipidemia and hypertension, we decided to adjust for all components of the metabolic syndrome including glucose. Moreover, there is no single clinical definition for the clustering of metabolic abnormalities. We used the ATPIII-definition of metabolic syndrome[4] because it is most commonly used in studies, best related with the development of vascular diseases and easy to use in clinical practice. However, we realize that there are more definitions for the metabolic syndrome[3].

In conclusion, in patients with manifest arterial disease without diabetes, insulin resistance clusters with the number of components of the metabolic syndrome, and elevated insulin resistance is independently associated with an increased risk for cardiovascular events. This relation can not be explained by the single components constituting the metabolic syndrome or by inflammation.

Competing interests

The authors declare that they have no competing interests regarding this study.

Authors' contributions

All authors have read and approved the final manuscript. SV carried out the analyses, had an important part in the conception of the present study, interpretation of the data and in writing the manuscript. YG participated in the design of the SMART-study and critically reviewed the analyses and the design of the present study. AW participated in performing the analyses and critically reviewed the manuscript. PG participated in the conception of the present study and made a substantial contribution in drafting the manuscript. FV made substantial contributions in interpretation of the data, conception of the present study and in writing the manuscript.

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REFERENCE LIST

1. Bonora E, Kiechl S, Willeit J, Oberhollenzer F, Egger G, Meigs JB, Bonadonna RC, Muggeo M: Insulin resistance as estimated by homeostasis model assessment predicts incident symptomatic cardiovascular disease in caucasian subjects from the general population: the Bruneck study. Diabetes Care 2007, 30:318-324.
2. Lakka HM, Lakka TA, Tuomilehto J, Salonen JT: Abdominal obesity is associated with increased risk of acute coronary events in men. Eur Heart J 2002, 23:706-713.
3. Eckel RH, Grundy SM, Zimmet PZ: The metabolic syndrome. Lancet 2005, 365:1415-1428.
4. Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, Gordon DJ, Krauss RM, Savage PJ, Smith SC, Jr., Spertus JA, Costa F: Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 2005, 112:2735-2752.
5. Bonora E, Formentini G, Calcaterra F, Lombardi S, Marini F, Zenari L, Saggiani F, Poli M, Perbellini S, Raffaelli A, Cacciatori V, Santi L, Targher G, Bonadonna R, Muggeo M: HOMA-estimated insulin resistance is an independent predictor of cardiovascular disease in type 2 diabetic subjects: prospective data from the Verona Diabetes Complications Study. Diabetes Care 2002, 25:1135-1141.
6. Adler AI, Levy JC, Matthews DR, Stratton IM, Hines G, Holman RR: Insulin sensitivity at diagnosis of Type 2 diabetes is not associated with subsequent cardiovascular disease (UKPDS 67). Diabet Med 2005, 22:306-311.
7. Hanley AJ, Williams K, Stern MP, Haffner SM: Homeostasis model assessment of insulin resistance in relation to the incidence of cardiovascular disease: the San Antonio Heart Study. Diabetes Care 2002, 25:1177-1184.
8. Hedblad B, Nilsson P, Engstrom G, Berglund G, Janzon L: Insulin resistance in non-diabetic subjects is associated with increased incidence of myocardial infarction and death. Diabet Med 2002, 19:470-475.
9. Meigs JB, Rutter MK, Sullivan LM, Fox CS, D'Agostino RB, Sr., Wilson PW: Impact of insulin resistance on risk of type 2 diabetes and cardiovascular disease in people with metabolic syndrome. Diabetes Care 2007, 30:1219-1225.
10. Resnick HE, Jones K, Ruotolo G, Jain AK, Henderson J, Lu W, Howard BV: Insulin resistance, the metabolic syndrome, and risk of incident cardiovascular disease in nondiabetic american indians: the Strong Heart Study. Diabetes Care 2003, 26:861-867.
11. Rutter MK, Meigs JB, Sullivan LM, D'Agostino RB, Sr., Wilson PW: Insulin resistance, the metabolic syndrome, and incident cardiovascular events in the Framingham Offspring Study. Diabetes 2005, 54:3252-3257.
12. Saely CH, Aczel S, Marte T, Langer P, Hoefle G, Drexel H: The metabolic syndrome, insulin resistance, and cardiovascular risk in diabetic and nondiabetic patients. J Clin Endocrinol Metab 2005, 90:5698-5703.
13. Tenenbaum A, Adler Y, Boyko V, Tenenbaum H, Fisman EZ, Tanne D, Lapidot M, Schwammenthal E, Feinberg MS, Matas Z, Morto M, Behar S: Insulin resistance is associated with increased risk of major cardiovascular events in patients with preexisting coronary artery disease. Am Heart J 2007, 153:559-565.
14. Yanase M, Takatsu F, Tagawa T, Kato T, Arai K, Koyasu M, Horibe H, Nomoto S, Takemoto K, Shimizu S, Watarai M: Insulin resistance and fasting hyperinsulinemia are risk factors for new cardiovascular events in patients with prior coronary artery disease and normal glucose tolerance. Circ J 2004, 68:47-52.
15. Gorter PM, Olijhoek JK, van der Graaf Y, Algra A, Rabelink TJ, Visseren FL: Prevalence of the metabolic syndrome in patients with coronary heart disease, cerebrovascular disease, peripheral arterial disease or abdominal aortic aneurysm. Atherosclerosis 2004, 173:363-369.

16. Olijhoek JK, van der Graaf Y, Banga JD, Algra A, Rabelink TJ, Visseren FL: The metabolic syndrome is associated with advanced vascular damage in patients with coronary heart disease, stroke, peripheral arterial disease or abdominal aortic aneurysm. Eur Heart J 2004, 25:342-348.

17. Martens FM, Visseren FL: The operative risk factors in the metabolic syndrome: is it lipids and high BP or are there direct vascular effects of insulin resistance and obesity. Curr Diab Rep 2007, 7:74-81.

18. Sakkinen PA, Wahl P, Cushman M, Lewis MR, Tracy RP: Clustering of procoagulation, inflammation, and fibrinolysis variables with metabolic factors in insulin resistance syndrome. Am J Epidemiol 2000, 152:897-907.

19. Scuteri A, Najjar SS, Muller DC, Andres R, Hougaku H, Metter EJ, Lakatta EG: Metabolic syndrome amplifies the age-associated increases in vascular thickness and stiffness. J Am Coll Cardiol 2004, 43:1388-1395.

20. Simons PC, Algra A, van de Laak MF, Grobbee DE, van der Graaf Y: Second manifestations of ARTerial disease (SMART) study: rationale and design. Eur J Epidemiol 1999, 15:773-781.

21. Wassink AM, van der Graaf Y, van Haeften TW, Spiering W, Soedamah-Muthu SS, Visseren FL: Waist circumference and metabolic risk factors have separate and additive effects on the risk of future Type 2 diabetes in patients with vascular diseases. A cohort study. Diabet Med 2011, 28:932-940.

22. Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC: Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985, 28:412-419.

23. Bonora E, Targher G, Alberiche M, Bonadonna RC, Saggiani F, Zenere MB, Monauni T, Muggeo M: Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity. Diabetes Care 2000, 23:57-63.

24. Haffner SM, Miettinen H, Stern MP: The homeostasis model in the San Antonio Heart Study. Diabetes Care 1997, 20:1087-1092.

25. Muniyappa R, Lee S, Chen H, Quon MJ: Current approaches for assessing insulin sensitivity and resistance in vivo: advantages, limitations, and appropriate usage. Am J Physiol Endocrinol Metab 2008, 294:E15-E26.

26. Caccamo G, Bonura F, Bonura F, Vitale G, Novo G, Evola S, Evola G, Grisanti MR, Novo S: Insulin resistance and acute coronary syndrome. Atherosclerosis 2010, 211:672-675.

27. Spencer M, Unal R, Zhu B, Rasouli N, McGehee RE, Jr., Peterson CA, Kern PA: Adipose Tissue Extracellular Matrix and Vascular Abnormalities in Obesity and Insulin Resistance. J Clin Endocrinol Metab 2011.

28. Pasarica M, Sereda OR, Redman LM, Albarado DC, Hymel DT, Roan LE, Rood JC, Burk DH, Smith SR: Reduced adipose tissue oxygenation in human obesity: evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. Diabetes 2009, 58:718-725.

29. van de Woestijne AP, Monajemi H, Kalkhoven E, Visseren FL: Adipose tissue dysfunction and hypertriglyceridemia: mechanisms and management. Obes Rev 2011, 12:829-840.

30. Khan AM, Cheng S, Magnusson M, Larson MG, Newton-Cheh C, McCabe EL, Coviello AD, Florez JC, Fox CS, Levy D, Robins SJ, Arora P, Bhasin S, Lam CS, Vasan RS, Melander O, Wang TJ: Cardiac natriuretic peptides, obesity, and insulin resistance: evidence from two community-based studies. J Clin Endocrinol Metab 2011, 96:3242-3249.

31. Dormandy JA, Charbonnel B, Eckland DJ, Erdmann E, Massi-Benedetti M, Moules IK, Skene AM, Tan MH, Lefebvre PJ, Murray GD, Standl E, Wilcox RG, Wilhelmsen L, Betteridge J, Birkeland K Golay A, Heine RJ, Koranyi L, Laakso M, Mokan M, Norkus A, Pirags V, Podar T Scheen A, Scherbaum W Schernthaner G Schmitz O Skrha J, Smith U, Taton J; PROactive investigators: Secondary prevention of macrovascular events in patients with type 2 diabetes in the PROactive Study (PROspective pioglitAzone Clinical Trial In macroVascular Events): a randomised controlled trial. Lancet 2005, 366:1279-1289.

32. Frye RL, August P, Brooks MM, Hardison RM, Kelsey SF, MacGregor JM, Orchard TJ, Chaitman BR, Genuth SM, Goldberg SH, Hlatky MA, Jones TL, Molitch ME, Nesto RW, Sako EY, Sobel BE: A randomized trial of therapies for type 2 diabetes and coronary artery disease. N Engl J Med 2009, 360:2503-2515.

33. Shin JY, Lee HR, Lee DC: Increased arterial stiffness in healthy subjects with high-normal glucose levels and in subjects with pre-diabetes. Cardiovasc Diabetol 2011, 10:30.

34. Gomez-Marcos MA, Recio-Rodriguez JI, Patino-Alonso MC, gudo-Conde C, Gomez-Sanchez L, Rodriguez-Sanchez E, Martin-Cantera C, Garcia-Ortiz L: Relationship between intima-media thickness of the common carotid artery and arterial stiffness in subjects with and without type 2 diabetes: a case-series report. Cardiovasc Diabetol 2011, 10:3.

35. Wheatcroft SB, Williams IL, Shah AM, Kearney MT: Pathophysiological implications of insulin resistance on vascular endothelial function. Diabet Med 2003, 20:255-268.

36. Wassink AM, van der Graaf Y, Olijhoek JK, Visseren FL: Metabolic syndrome and the risk of new vascular events and all-cause mortality in patients with coronary artery disease, cerebrovascular disease, peripheral arterial disease or abdominal aortic aneurysm. Eur Heart J 2008, 29:213-223.

37. Tenenbaum A, Fisman EZ: "The metabolic syndrome... is dead": these reports are an exaggeration. Cardiovasc Diabetol 2011, 10:11.

38. Faber DR, de Groot PG, Visseren FL: Role of adipose tissue in haemostasis, coagulation and fibrinolysis. Obes Rev 2009.

39. Trovati M, Anfossi G, Cavalot F, Massucco P, Mularoni E, Emanuelli G: Insulin directly reduces platelet sensitivity to aggregating agents. Studies in vitro and in vivo. Diabetes 1988, 37:780-786.

40. Kim JA, Montagnani M, Koh KK, Quon MJ: Reciprocal relationships between insulin resistance and endothelial dysfunction: molecular and pathophysiological mechanisms. Circulation 2006, 113:1888-1904.

41. Goff DC, Jr., D'Agostino RB, Jr., Haffner SM, Otvos JD: Insulin resistance and adiposity influence lipoprotein size and subclass concentrations. Results from the Insulin Resistance Atherosclerosis Study. Metabolism 2005, 54:264-270.

42. DeFronzo RA, Tobin JD, Andres R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol 1979, 237:E214-E223.

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Chapter 8

C-reactive protein increases the risk of incident type 2 diabetes in patients with manifest arterial disease; The SMART study

Submitted

ABSTRACT

Introduction: Systemic low-grade inflammation, as measured by high sensitive C-reactive protein (hsCRP) may contribute to the risk of type 2 diabetes in patients with manifest arterial disease.
Methods: Cohort study in 4072 patients with manifest arterial disease without diabetes. The relation between quartiles of hsCRP and type 2 diabetes was assessed with Cox regression analyses, taking age, smoking and blood pressure- and lipid-lowering medication into account. Insulin resistance was estimated with homeostasis model of insulin resistance (HOMA-IR). In exploratory models adjustments were performed for BMI and visceral and subcutaneous adipose tissue thickness.
Results: During a median follow up of 5.0 (IQR 2.5-8.2) years 288 subjects developed diabetes. High hsCRP was independently associated with incident diabetes (Q4 vs Q1 males: HR 1.62; 95%CI 1.06-2.48; females: HR 3.12; 95%CI 1.57-6.21). HOMA-IR at baseline is related to hsCRP plasma levels (Q4 vs Q1: males: β 0.27; 95%CI 0.19-0.36; females: β 0.35; 95%CI 0.22-0.48). The risk of diabetes associated with hsCRP was abolished in males (Q4 vs 1 HR 1.23; 95%CI 0.80-1.88) and attenuated in females (Q4 vs 1 HR 2.32; 95%CI 1.14-4.75) after adding BMI to the model, but not modified by statin use (p for interaction: 0.61).
Conclusions: Patients with manifest arterial disease with high hsCRP plasma levels are at increased risk to develop type 2 diabetes and are more insulin resistant as compared to those with low hsCRP levels. This increase in risk is more pronounced in females than in males and is not modified by statin use.

INTRODUCTION

Type 2 diabetes is a multifactorial disease characterized by metabolic disturbances in carbohydrate, lipid and protein metabolism due to insulin resistance and relative insufficiency of pancreatic beta cell insulin secretion. Insulin resistance is highly associated with abdominal obesity[1]. An increase in visceral adipose tissue (VAT) causes changes in the function of adipocytes and macrophages, also known as adipose tissue dysfunction, which induces a state of low grade inflammation[2]. Dysfunctional adipose tissue produces large amounts of free fatty acids and in addition it is able to secrete several pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), and interleukin 6 (IL-6[3;4]). Activation of toll-like receptor-4 on adipocytes by free fatty acids further increases the production of pro-inflammatory cytokines and also induces insulin resistance by affecting the intracellular insulin signaling cascade[5]. Thus, insulin resistance is the direct result of adipose tissue dysfunction and inflammation and is related to increased risk of type 2 diabetes and atherosclerosis. Therefore, obesity-induced inflammation is thought to be a 'common soil' underlying both cardiovascular disease and type 2 diabetes[6].

Plasma CRP is an acute phase protein, mainly produced by hepatocytes in response to IL-6[7], although also adipocytes are able to secrete CRP[3]. Plasma hsCRP is a predictor and a risk factor for cardiovascular events[8]. In addition, studies have shown that CRP is related to an increased risk of incident type 2 diabetes[9-19]. Whether this relation is also present in patients with clinically manifest arterial disease is unknown. The use of statins is very high in this group of patients. Statins have anti-inflammatory properties as measured by reduced hsCRP levels during statin treatment[20]. Moreover, statins are associated with a slightly increased risk of type 2 diabetes[21].

In the present study we investigated the relation between inflammation, as reflected by plasma high sensitive CRP (hsCRP) levels, and the risk of incident type 2 diabetes in patients with clinically manifest arterial disease. In addition, it was determined whether this relation was driven by obesity and whether hsCRP is associated with insulin resistance at baseline. Finally, the relation between hsCRP and incident type 2 diabetes was assessed in patients with and without statin therapy.

Chapter 8

METHODS

Study settings, participants and design

Subjects were all enrolled in the Second Manifestations of Arterial disease (SMART) study. The SMART study is an ongoing prospective single-centre cohort study in patients with manifest arterial disease or cardiovascular risk factors.[22] The Medical Ethics Committee approved the study, and all participants gave their written informed consent. The rationale and design of the SMART study and a detailed description of the criteria used to define the different manifest arterial diseases were published previously[22].

The data of the 5788 participants included in the cohort in the period September 1st, 1996 to March 1st, 2010, with clinical manifestations of arterial disease (coronary heart disease, cerebrovascular disease, peripheral arterial disease or abdominal aortic aneurysm) were considered. Patients who had died or were lost to follow up (n=577) before the assessment of incident T2DM started in 2006 (see: follow up) were excluded. Patients with diabetes, defined as a referral diagnosis of diabetes, self-reported diabetes or use of glucose-lowering agents (type 1 or 2) at study inclusion (n=889)

were excluded. If glucose was ≥7 mmol/l without a history of diabetes, patients were only included if they were not on glucose-lowering therapy after 1 year follow up[23]. In addition, patients with hsCRP levels >15mg/l (n=196) or missing data on hsCRP (n=54) were excluded, leaving 4072 participants for analyses. For analyses with HOMA-IR as outcome, subjects included in the cohort after August 2003 were considered (n=2482), since plasma insulin levels were measured since that date.

Data acquisition

All baseline measurements were performed on a single day at the UMC Utrecht. Medical history, use of current medication and current and past cigarette smoking behavior were derived from a questionnaire described elsewhere[22]. Height, weight, waist circumference and blood pressure were measured. Blood samples were collected after an overnight fast. Serum hsCRP was measured by immunonephelometry (Nephelometer Analyzer BN II, Dade-Behring, Germany) with a lower detection limit of 0.1 mg/l. HsCRP measurements below the lower limit of the detection limit of 0.1 mIU/l (n=11) were set at 0.1mIU/l.

HOMA-IR was used as quantitative estimate of the degree of insulin resistance at baseline. Plasma insulin was measured with an immunometric technique on an IMMULITE 1000 Analyzer (Diagnostic Products Corporation, Los Angeles, USA). Insulin measurements below the lower limit of detection of 2 mIU/l (n=74) were set at 2 mIU/l. The value for insulin resistance can be assessed by the formula: HOMA-IR = (fasting serum glucose (mmol/l) x fasting serum insulin (mIU/l) / 22.5)[24]. The Adult Treatment Panel (ATP) III criteria were taken for the definition of the metabolic syndrome[25].

Follow up

The main outcome of interest for this study was incident type 2 diabetes. In order to assess the incidence of diabetes, all patients that had been included until June 2006 without diabetes at baseline received a questionnaire in the period between June and December 2006 to assess the incidence of type 2 diabetes after study inclusion. After 2006, all patients were biannually asked to complete this questionnaire. Patients were asked whether they had diabetes and if 'yes', they received a supplementary questionnaire regarding date of diagnosis, initial and current treatment (oral medication or insulin) and family history of diabetes. Patients and/or their general practitioners were contacted by telephone for further information if the answers were incomplete or unclear and also non-responders were contacted. All diabetes cases were audited and classified by two independent physicians. Cross validation with the hospital diagnosis registry revealed that none of the patients who reported not to have diabetes had a physician's diagnosis of diabetes. Duration of follow-up was defined as the period between the date of study inclusion and the date of incident type 2 diabetes, date of loss to follow-up or the pre-selected closing date of March 1st, 2010. From 1996 until 1 March 2010, 57 out of 4072 patients (2%) were lost to follow-up.

Data analyses

Continuous variables are expressed as mean ± standard deviation (SD) when normally distributed or as median with interquartile range (IQR) if the distribution was skewed. Single imputation methods were used to reduce missing covariate data for smoking (n= 13), BMI (n= 3), VAT thickness (n= 7) and subcutaneous adipose tissue (SAT) thickness (n= 26), since incomplete case analysis leads to loss of statistical power and possibly bias.

The relation between hsCRP and incident type 2 diabetes was quantified with Cox proportional hazards analysis and results are expressed as hazard ratios (HR) with corresponding 95% confidence intervals (CI). Patients were censored if they died or were lost to follow-up. The proportional hazard assumption was confirmed by testing the correlations between scaled Schoenfeld residuals for hsCRP and time. No significant non-proportionality ($p<0.05$) was observed.

To investigate whether the relation between hsCRP and incident type 2 diabetes was modified by gender, body mass index (BMI) or statin therapy, interaction terms for these variables were included in the Cox proportional hazard model. Only gender appeared to modify the relation between hsCRP and incident type 2 diabetes significantly (p-value of the interaction term 0.03), analyses were stratified according to gender. BMI (p-value 0.27) and statin therapy (p-value 0.61) were not significant effect modifiers.

Crude estimations of the relation between hsCRP and incident type 2 diabetes are shown in model 1. In model 2 adjustments were made for age and gender and in model 3 additional adjustments were made for current smoking, use of lipid lowering medication and use of blood pressure lowering medication, all of which are considered as potential confounding factors in the relation between hsCRP and incident type 2 diabetes. Conclusions of the study are based on model 3. Finally, in exploratory models, an additional adjustment was made for BMI, which may be in the causal pathway of the relation between hsCRP and incident type 2 diabetes.

Next, linear regression analyses were performed to estimate the relation between hsCRP and HOMA-IR. Results are expressed as beta regression coefficients with corresponding 95% CI's. Again, analyses were conducted with a crude model (model 1) and two additional models (models 2 and 3) to adjust for the same potential confounding factors as in the Cox proportional hazard analysis. In exploratory models, additional adjustments were made for measures of obesity, which may be in the causal pathway of the relation between hsCRP and HOMA-IR, being BMI, VAT thickness and SAT tissue thickness. Differences between patients with and without statin therapy were explored with Chi Square tests, T-tests and Kruscal Wallis tests for categorical, normally distributed and skewed variables respectively.

Chapter 8

RESULTS

Study Population

Out of the 4072 subjects with clinically manifest arterial disease, 2976 subjects were male (73%) and mean age was 60 ± 10 years. Median hsCRP was 1.7 (IQR 0.9-3.5) in men and 2.0 (IQR 1.0-4.2) in women. BMI was 26.7 ± 3.5 in males and 26.3 ± 4.5 in females. Baseline characteristics across quartiles of hsCRP are presented in table 1.

Follow-up and incident type 2 diabetes

During a median follow up of 5.0 (IQR 2.5-8.2) years and total follow up of 21,845 person years, 207 (7%) males and 79 (7%) females developed type 2 diabetes, corresponding with an incidence rate of 12.8 per 1000 patient years (95%CI 11.2-14.7) in males and 13.8 per 1000 person years (95%CI 10.9-17.2) in females. The incidence of type 2 diabetes increased with increasing hsCRP (incidence rate per 100 person years: Q1: 8.9 95%CI 6.6-11.8, Q2: 9.4 95%CI 7.0-12.4, Q3 15.7 95%CI 12.6-19.3, Q4 17.5 95%CI 14.3-21.3)

HsCRP and risk of type 2 diabetes

Subjects with elevated hsCRP levels were at increased risk to develop type 2 diabetes independent of possible confounding factors (table 2). This increased risk for subjects in the highest quartile of hsCRP was more pronounced in females (Q4 vs. Q1 HR 3.12; 95%CI 1.57-6.21) than in males (Q4 vs. Q1 HR 1.62; 95%CI 1.06-2.48) (p for trend in females <0.001; p for trend in males 0.006), based on the fully adjusted model (model 3). Exploratory analyses revealed that the relation between hsCRP and incident type 2 diabetes was attenuated in females and abolished in males when adding BMI to the model (Q4 4 vs. Q1 HR 2.32; 95%CI 1.14-4.75 in females and Q4 vs. Q1 HR 1.23; 95%CI 0.80-1.88 in males) (p for trend females 0.004; males 0.17) (table 2).

Tabel 1 | Baseline characteristics according to quartiles of hsCRP*

hsCRP (n=4072)	**Quartile 1** n=985	**Quartile 2** n=1075	**Quartile 3** n=1000	**Quartile 4** n=1012
hsCRP range (mg/l)	0.1-1.0	0.9-2.0	1.7-4.2	3.5-15.0
hsCRP mean (mg/l)	0.5±0.2	1.3±0.3	2.6±0.6	6.7±2.8
Age (years)	58±10	59±11	60±10	59±10
Male gender, n (%)	742 (73)	754 (73)	735 (73)	745 (73)
Current smoking, n (%)	223 (22)	309 (30)	355 (35)	454 (45)
Cerebrovascular disease, n (%)	286 (28)	275 (27)	277 (28)	280 (28)
Coronary heart disease, n (%)	663 (66)	687 (66)	620 (62)	538 (53)
Peripheral arterial disease, n (%)	106 (11)	145 (14)	187 (19)	267 (26)
Abdominal aortic aneurysm, n (%)	33 (3)	51 (5)	82 (8)	122 (12)
Lipid-lowering agents, n (%)	752 (74)	721 (70)	655 (65)	557 (55)
Anti-platelet agents, n (%)	821 (81)	825 (80)	746 (74)	690 (68)
Blood pressure-lowering agents, n (%)	732 (72)	774(75)	746 (74)	706 (70)
Body mass index (kg/m^2)	25.4±3.3	26.5±3.6	27.2±3.8	27.1±4.1
Waist circumference (cm)	90.3±10.8	94.0±11.3	96.5±11.3	96.7±11.8
Abdominal adipose tissue (cm)	8.0±2.2	8.8±2.4	9.3±2.4	9.5±2.5
Blood pressure systolic (mmHg)	138±21	139±21	141±22	142±23
Blood pressure diastolic (mmHg)	82±11	82±11	82±11	83±12
Fasting glucose (mmol/l)	5.6±0.7	5.7±0.7	5.8±0.8	5.8±0.8
Triglycerides (mmol/l)	1.2 (0.9-1.6)	1.4 (1.0-2.0)	1.4 (1.1-2.1)	1.5 (1.1-2.2)
HDL-cholesterol (mmol/l)	1.34±0.4	1.26±0.38	1.25±0.37	1.17±0.35
LDL-cholesterol (mmol/l)	2.5 (2.1-3.2)	2.7 (2.1-3.5)	2.9 (2.3-3.7)	3.1 (2.5-3.9)
Creatinin clearance MDRD (ml/min/1.73m^2)	78±15	78±17	76±17	75±17
Fasting insulin (mIU/l)	8.8±5.2	10.6±7.3	11.4±7.6	11.8±9.4
HOMA-IR	1.9 (1.2-2.9)	2.3 (1.5-3.4)	2.4 (1.5-3.7)	2.5 (1.6-3.7)
Metabolic syndrome (ATPIII), n (%)	288 (29)	422 (41)	443 (44)	468 (46)
Urine albumin/creatinin ratio >3.0 mg/mmol, n (%)	109 (11)	125 (12)	147 (15)	182 (18)

* Quartiles of CRP are sex-pooled

Relation between hsCRP and HOMA-IR

At baseline, hsCRP was associated with insulin resistance, as measured with HOMA-IR (table 3). Males in Q4 of hsCRP had 0.27 units (β 0.27; 95%CI 0.19-0.36) increase of HOMA-IR on a logarithmic scale as compared to Q1 of hsCRP (model 3). For females the increase in HOMA-IR in the highest quartile compared to the lowest quartile was 0.35 units (β 0.35; 95%CI 0.22-0.48). The relation between hsCRP and HOMA-IR was attenuated after additional adjustment for BMI (Q4 vs. Q1 β 0.16; 95%CI 0.04-0.29 in females and Q4 vs. Q1 β 0.12; 95%CI 0.04-0.21 in males) or VAT thickness (Q4 vs. Q1: β 0.13; 95%CI 0.01-0.26 in females and Q4 vs Q1 β 0.11; 95%CI 0.03-0.19 in males). Additional adjustment for SAT thickness did not attenuate the results (Q4 vs. Q1 β 0.28; 95%CI 0.15-0.41 in females and Q4 vs. Q1 β 0.27; 95%CI 0.19-0.36 in males).

Table 2 | hsCRP and the risk of new onset type 2 diabetes stratified for gender

hsCRP	Quartile 1		Quartile 2		Quartile 3		Quartile 4	
Model	**# events**	**HR (95%CI)**	**# events**	**HR (95%CI)**	**# events**	**HR (95%CI)**	**# events**	**HR (95%CI)**
Male gender (n= 2976)								
I	34	1 (reference)	39	1.07 (0.68-1.70)	67	1.71 (1.13-2.58)	67	1.60 (1.07-2.44)
II		1 (reference)		1.07 (0.68-1.70)		1.69 (1.12-2.55)		1.60 (1.05-2.41)
III		1 (reference)		1.08 (0.68-1.71)		1.69 (1.11-2.57)		1.62 (1.06-2.48)
Female gender (n=1096)								
I	11	1 (reference)	11	0.99 (0.43-2.28)	21	1.79 (0.86-2.71)	36	2.97 (1.51-5.85)
II		1 (reference)		0.95 (0.41-2.19)		1.71 (0.82-3.55)		2.99 (1.52-5.88)
III		1 (reference)		0.95 (0.41-2.20)		1.70 (0.82-3.55)		3.12 (1.57-6.21)

Model I: crude model
Model II: adjusted for age
Model III: model II additionally adjusted for lipid lowering medication, blood pressure lowering medication and smoking

Table 3 | hsCRP in relation to insulin resistance (logtransformed HOMA-IR) stratified for gender

hsCRP	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Model	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**	**β (95%CI)**
Male gender (n=2976)				
I	0 (reference)	0.19 (0.10-0.28)	0.32 (0.23-0.41)	0.26 (0.17-0.35)
II	0 (reference)	0.19 (0.10-0.28)	0.33 (0.24-0.42)	0.26 (0.17-0.35)
III	0 (reference)	0.18 (0.09-0.27)	0.31 (0.22-0.40)	0.27 (0.19-0.36)
Female gender (n=1096)				
I	0 (reference)	0.15 (0.02-0.29)	0.21 (0.08-0.34)	0.35 (0.22-0.48)
II	0 (reference)	0.14 (0.01-0.28)	0.20 (0.07-0.33)	0.35 (0.21-0.48)
III	0 (reference)	0.12 (-0.01-0.25)	0.21 (0.08-0.34)	0.35 (0.22-0.48)

Model I: crude model
Model II: adjusted for age
Model III: model II additionally adjusted for lipid lowering medication, blood pressure lowering medication and smoking

Influence of statin therapy on the relation between hsCRP and incident type 2 diabetes

Median hsCRP was 1.6 mg/l (IQR: 0.8-3.2) in patients on statin therapy and 2.3 mg/l (IQR: 1.1-4.6) in patients not on statin therapy (p<0.001). Metabolic syndrome was more prevalent (44% vs. 32%) (p<0.001) and insulin resistance was more prominent in those on statin therapy (median HOMA-IR 2.0; IQR: 1.3-3.0 vs. 2.3; IQR: 1.5-3.6) (p<0.001). The incidence rate of type 2 diabetes was 13.8 (95%CI 11.8-16.1) per 1000 person years in patients on statin therapy and 12.2 (95%CI 10.0-14.6) per 1000 person years in patients not on statin therapy. The increased risk of incident type 2 diabetes in subjects with high hsCRP levels was comparable in patients on statin therapy (Q4 vs. 1 HR 2.35; 95%CI 1.44-3.83) and those not on statin therapy (Q4 vs. Q1 HR 1.75; 95%CI 1.02-3.03; p-value for interaction: 0.61) (table 4).

Table 4 | hsCRP and the risk of new onset type 2 diabetes in patients with and without statin use

hsCRP	Quartile 1		Quartile 2		Quartile 3		Quartile 4	
Model	**# events**	**HR (95%CI)**	**# events**	**HR (95%CI)**	**# events**	**HR (95%CI)**	**# events**	**HR (95%CI)**
No statin use (n=1406)								
I	20	1 (reference)	25	1.19 (0.66-2.14)	32	1.52 (0.87-2.66)	41	1.92 (1.13-3.28)
II		1 (reference)		1.14 (0.63-2.07)		1.44 (0.82-2.54)		1.86 (1.09-3.18)
III		1 (reference)		1.13 (0.63-2.05)		1.32 (0.75-2.33)		1.75 (1.02-3.03)
Statin use (n=2666)								
I	23	1 (reference)	34	1.46 (0.86-2.47)	52	2.09 (1.28-3.41)	59	2.39 (1.48-3.88)
II		1 (reference)		1.45 (0.85-2.46)		2.07 (1.27-3.38)		2.48 (1.47-3.86)
III		1 (reference)		1.44 (0.85-2.45)		2.05 (1.25-3.36)		2.35 (1.44-3.83)

Model I: crude model
Model II: adjusted for age
Model III: model II additionally adjusted for blood pressure lowering medication and smoking

DISCUSSION

In the present study it is shown that an increase in hsCRP is an independent risk factor for developing type 2 diabetes in patients with clinically manifest arterial disease. In addition, a positive relation was found between hsCRP and insulin resistance as measured with HOMA-IR. The hsCRP associated risk for type 2 diabetes was higher in women compared to men and was present irrespective of the use of statins.

In the general population the risk for type 2 diabetes associated with elevated hsCRP levels is higher than the risk found in the present study in patients with manifest arterial disease [9-12;14;15;18;26-30]. These studies report increases in risk for type 2 diabetes with high CRP of more than 5-fold compared to a 2-3-fold increase in the present study. This may be due to the high prevalence of other risk factors for type 2 diabetes, clustered in the metabolic syndrome in our study population. These risk factors and other unknown risk factor are competing for the risk of type 2 diabetes. It may be possible that the risk of developing type 2 diabetes that can be attributed to hsCRP is lower than in other studies

due to presence of these competing risk factors. Only in a nested case control study conducted in men and women aged 40-74 years, a lower risk of type 2 diabetes associated with hsCRP was found than in our study[15]. In that study, cases were matched to controls on the basis of BMI, hereby taking away the influence of one of the possible factors in the causal pathway of hsCRP and type 2 diabetes. In the present study, the increased risk of type 2 diabetes associated with higher hsCRP levels was attenuated to a large extent by adding BMI to the regression model, indicating that BMI is part of the causal pathway between hsCRP and incident type 2 diabetes. The association between hsCRP and HOMA-IR was attenuated after adjustment for BMI as well as after adjustment for VAT thickness. This attenuation was not present when adjusting for SAT thickness, confirming the functional differences between VAT and SAT in relation to inflammation and insulin resistance [31]. VAT, more than SAT, is known to be associated with CRP[31] and type 2 diabetes[23].

Our findings show that the relation between hsCRP and HOMA-IR was modified by gender, the association being stronger in women than in men. This is in concordance with previous studies that have found a stronger association between inflammation and insulin resistance in females compared to males[30] and with studies that have reported inflammation to be a stronger causative factor for type 2 diabetes in females than in males[19]. A difference in body composition and functional differences in adipose tissue in men and women may be the underlying cause for these gender differences in the relation between inflammation and insulin resistance. Also, the same amount of adipose tissue increased hsCRP levels more in women than in men, indicated by the stronger association between visceral adipose tissue and hsCRP in women than in men, pointing towards functional differences in adipose tissue[31].

Data presented in the present study show that the risk of developing type 2 diabetes is high in patients with clinically manifest atherosclerosis, as indicated by the incidence rate of 13 per 1000 patient years compared to 9.2 per 1000 patient years in a healthy population of similar age[11], although also a higher incidence rate of 16.5% has been reported in the general population[10]. This high risk of developing type 2 diabetes together with the notion that type 2 diabetes confers an increased risk for new vascular events, underlines the importance of prevention of type 2 diabetes in these patients. Patients with manifest atherosclerosis are already at high vascular risk and the development of type 2 diabetes would put them at even higher risk[32;33]. Women in the highest quartile of hsCRP had a 3.1-fold higher risk of developing type 2 diabetes compared to women in the lowest quartile and this risk was 1.6-fold higher in men in the highest compared to the lowest quartile of hsCRP. This indicates that in a cohort of patients at high risk of future type 2 diabetes, hsCRP is able to identify those at the highest risk for developing type 2 diabetes. Epidemiologic studies, including the current study, have designated CRP as a risk marker for type 2 diabetes, but CRP may also play a causal role in the development of type 2 diabetes by influencing insulin signalling[34;35]. CRP induces phosphorylation of insulin receptor substrate (IRS)-1 in myocytes in vitro, hereby impairing the insulin signaling pathway that promotes glucose transport[34].

HsCRP levels in the current study were relatively low compared to the reported hsCRP levels in studies in the general population, roughly ranging from a median of 1.7 to 4.7 mg/dl[9-12;14;15;17-19]. As hsCRP levels are at least partially driven by abdominal obesity[31], a low BMI would be an explanation for this. However, BMI was only slightly lower in our study (26.7 kg/m^2 in males and 26.3 kg/m^2 in females) compared to other studies. Furthermore, statins lower CRP independent from LDL-reduction[36]. There was a high level of statin use in our population, which was not common practice or not reported in study populations with high CRP levels[10;15]. From the data of five large trials it

was concluded that statin therapy results in a small increase in diabetes risk[21]. In our study, there was no effect modification of statin therapy on the relation between hsCRP and incident type 2 diabetes. Incidence rates of type 2 diabetes were equal in patients with and without statin therapy. Furthermore, the CRP associated risk of type 2 diabetes was similar in patients using statins compared to the risk in patients not using statins. Thus, although statins are known to reduce hsCRP, there was no influence of statins on the incidence rate of type 2 diabetes nor did statin use influence the hsCRP-associated risk of type 2 diabetes. The absence of influence of statins on the relation between hsCRP and incident of type 2 diabetes may be due to the non-experimental design of the SMART study. Confounding by indication may be an important factor in interpreting the results of observational studies. Possibly statins are prescribed to those with the highest risk of type 2 diabetes or to those with high CRP levels. In the present study statin use was more prevalent in patients in the highest quartile of CRP.

One of the strengths of this study is that it was conducted in a large cohort of patients with various clinical manifestations of atherosclerosis. The study population is relevant in clinical practice, because type 2 diabetes prevalence is high in patients with manifest arterial diseases[37]. We also acknowledge limitations of our study. Part of the outcome of type 2 diabetes is determined retrospectively. However, the response rate of the questionnaire was very high (98%), and answers were checked with the hospital registry. Furthermore, only hsCRP was measured as a marker of inflammation, whereas other inflammatory markers such as TNF-α and IL-6 may also affect insulin resistance thus may influence type 2 diabetes incidence.

In conclusion, patients with manifest arterial disease and high plasma hsCRP levels are at increased risk to develop type 2 diabetes and are more insulin resistant as compared to patients with low hsCRP levels. This risk is higher in women than in men and is not modified by the use of statins.

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REFERENCE LIST

1 Campbell PJ, Gerich JE. Impact of obesity on insulin action in volunteers with normal glucose tolerance: demonstration of a threshold for the adverse effect of obesity. J Clin Endocrinol Metab 1990;70(4):1114-8.

2 Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 2008;29(24):2959-71.

3 Calabro P, Chang DW, Willerson JT, Yeh ET. Release of C-reactive protein in response to inflammatory cytokines by human adipocytes: linking obesity to vascular inflammation. J Am Coll Cardiol 2005;46(6):1112-3.

4 Fain JN, Madan AK, Hiler ML, Cheema P, Bahouth SW. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology 2004;145(5):2273-82.

5 Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest 2006;116(11):3015-25.

6 Stern MP. Diabetes and cardiovascular disease. The "common soil" hypothesis. Diabetes 1995;44(4):369-74.

7 Marinkovic S, Jahreis GP, Wong GG, Baumann H. IL-6 modulates the synthesis of a specific set of acute phase plasma proteins in vivo. J Immunol 1989;142(3):808-12.

8 Kaptoge S, Di AE, Lowe G, Pepys MB, Thompson SG, Collins R, et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. Lancet 2010;375(9709):132-40.

9 Barzilay JI, Abraham L, Heckbert SR, Cushman M, Kuller LH, Resnick HE, et al. The relation of markers of inflammation to the development of glucose disorders in the elderly: the Cardiovascular Health Study. Diabetes 2001;50(10):2384-9.

10 Bertoni AG, Burke GL, Owusu JA, Carnethon MR, Vaidya D, Barr RG, et al. Inflammation and the incidence of type 2 diabetes: the Multi-Ethnic Study of Atherosclerosis (MESA). Diabetes Care 2010;33(4):804-10.

11 Dehghan A, Kardys I, de Maat MP, Uitterlinden AG, Sijbrands EJ, Bootsma AH, et al. Genetic variation, C-reactive protein levels, and incidence of diabetes. Diabetes 2007;56(3):872-8.

12 Duncan BB, Schmidt MI, Pankow JS, Ballantyne CM, Couper D, Vigo A, et al. Low-grade systemic inflammation and the development of type 2 diabetes: the atherosclerosis risk in communities study. Diabetes 2003;52(7):1799-805.

13 Festa A, D'Agostino R, Jr., Tracy RP, Haffner SM. Elevated levels of acute-phase proteins and plasminogen activator inhibitor-1 predict the development of type 2 diabetes: the insulin resistance atherosclerosis study. Diabetes 2002;51(4):1131-7.

14 Freeman DJ, Norrie J, Caslake MJ, Gaw A, Ford I, Lowe GD, et al. C-reactive protein is an independent predictor of risk for the development of diabetes in the West of Scotland Coronary Prevention Study. Diabetes 2002;51(5):1596-600.

15 Lee CC, Adler AI, Sandhu MS, Sharp SJ, Forouhi NG, Erqou S, et al. Association of C-reactive protein with type 2 diabetes: prospective analysis and meta-analysis. Diabetologia 2009;52(6):1040-7.

16 Liu S, Tinker L, Song Y, Rifai N, Bonds DE, Cook NR, et al. A prospective study of inflammatory cytokines and diabetes mellitus in a multiethnic cohort of postmenopausal women. Arch Intern Med 2007;13;167(15):1676-85.

17 Pradhan AD, Manson JE, Rifai N, Buring JE, Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. JAMA 2001;18;286(3):327-34.

18 Tabak AG, Kivimaki M, Brunner EJ, Lowe GD, Jokela M, Akbaraly TN, et al. Changes in C-reactive protein levels before type 2 diabetes and cardiovascular death: the Whitehall II study. Eur J Endocrinol 2010;163(1):89-95.

19 Thorand B, Baumert J, Kolb H, Meisinger C, Chambless L, Koenig W, et al. Sex differences in the prediction of type 2 diabetes by inflammatory markers: results from the MONICA/KORA Augsburg case-cohort study, 1984-2002. Diabetes Care 2007;30(4):854-60.

20 Nissen SE, Tuzcu EM, Schoenhagen P, Crowe T, Sasiela WJ, Tsai J, et al. Statin therapy, LDL cholesterol, C-reactive protein, and coronary artery disease. N Engl J Med 2005;352(1):29-38.

21 Rajpathak SN, Kumbhani DJ, Crandall J, Barzilai N, Alderman M, Ridker PM. Statin therapy and risk of developing type 2 diabetes: a meta-analysis. Diabetes Care 2009;32(10):1924-9.

22 Simons PC, Algra A, van de Laak MF, Grobbee DE, van der Graaf Y. Second manifestations of ARTerial disease (SMART) study: rationale and design. Eur J Epidemiol 1999;15(9):773-81.

23 Wassink AM, van der Graaf Y, van Haeften TW, Spiering W, Soedamah-Muthu SS, Visseren FL. Waist circumference and metabolic risk factors have separate and additive effects on the risk of future Type 2 diabetes in patients with vascular diseases. A cohort study. Diabet Med 2011;28(8):932-40.

24 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28(7):412-9.

25 Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA, et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 2005;112(17):2735-52.

26 Doi Y, Kiyohara Y, Kubo M, Ninomiya T, Wakugawa Y, Yonemoto K, et al. Elevated C-reactive protein is a predictor of the development of diabetes in a general Japanese population: the Hisayama Study. Diabetes Care 2005 Oct;28(10):2497-500.

27 Han TS, Sattar N, Williams K, Gonzalez-Villalpando C, Lean ME, Haffner SM. Prospective study of C-reactive protein in relation to the development of diabetes and metabolic syndrome in the Mexico City Diabetes Study. Diabetes Care 2002;25(11):2016-21.

28 Hu FB, Meigs JB, Li TY, Rifai N, Manson JE. Inflammatory markers and risk of developing type 2 diabetes in women. Diabetes 2004;53(3):693-700.

29 Thorand B, Lowel H, Schneider A, Kolb H, Meisinger C, Frohlich M, et al. C-reactive protein as a predictor for incident diabetes mellitus among middle-aged men: results from the MONICA Augsburg cohort study, 1984-1998. Arch Intern Med 2003;163(1):93-9.

30 Thorand B, Baumert J, Doring A, Herder C, Kolb H, Rathmann W, et al. Sex differences in the relation of body composition to markers of inflammation. Atherosclerosis 2006;184(1):216-24.

31 Faber DR, van der Graaf Y, Westerink J, Visseren FL. Increased visceral adipose tissue mass is associated with increased C-reactive protein in patients with manifest vascular diseases. Atherosclerosis 2010;212(1):274-80.

32 Becker A, Bos G, de VF, Kostense PJ, Dekker JM, Nijpels G, et al. Cardiovascular events in type 2 diabetes: comparison with nondiabetic individuals without and with prior cardiovascular disease. 10-year follow-up of the Hoorn Study. Eur Heart J 2003;24(15):1406-13.

33 Hu FB, Stampfer MJ, Solomon CG, Liu S, Willett WC, Speizer FE, et al. The impact of diabetes mellitus on mortality from all causes and coronary heart disease in women: 20 years of follow-up. Arch Intern Med 2001;161(14):1717-23.

34 D'Alessandris C, Lauro R, Presta I, Sesti G. C-reactive protein induces phosphorylation of insulin receptor substrate-1 on Ser307 and Ser 612 in L6 myocytes, thereby impairing the insulin signalling pathway that promotes glucose transport. Diabetologia 2007;50(4):840-9.

35 Xu JW, Morita I, Ikeda K, Miki T, Yamori Y. C-reactive protein suppresses insulin signaling in endothelial cells: role of spleen tyrosine kinase. Mol Endocrinol 2007;21(2):564-73.

36 Ridker PM, Rifai N, Clearfield M, Downs JR, Weis SE, Miles JS, et al. Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events. N Engl J Med 2001;344(26):1959-65.

37 Wassink AM, van der Graaf Y, Soedamah-Muthu SS, Spiering W, Visseren FL. Metabolic syndrome and incidence of type 2 diabetes in patients with manifest vascular disease. Diab Vasc Dis Res 2008 ;5(2):114-22.

Chapter 9

General discussion

Being overweight is not inevitably detrimental[1;2]. However, the majority of obese subjects will develop metabolic disturbances such as insulin resistance[3-5]. A state of low grade inflammation associated with excess adipose tissue*6* is thought to be the culprit in the increase in atherosclerotic disease that accompanies obesity*7-9*. Adipocytes, macrophages, and endothelial cells in adipose tissue are able to secrete proteins that are involved in chemotaxis of immune cells and in coagulation[10;11]. Pro- and anti-inflammatory cytokines, secreted by adipocytes are referred to as adipokines. Increased secretion of these adipokines and cytokines accompanied by the excessive release of free fatty acids is referred to as adipose tissue dysfunction[10].

PART 1

Adipose tissue dysfunction in perivascular adipose tissue

The adipose tissue around the heart, epicardial adipose tissue, is able to secrete adipokines and cytokines in the same manner as visceral and subcutaneous adipose tissue[12]. Even higher levels of interleukin(IL)- 1β, IL-6 and tumor necrosis factor(TNF)-α mRNA were observed in epicardial adipose tissue than in subcutaneous adipose tissue of the same patient12. Just like subcutaneous adipose tissue, increased production of adipocytokines comes with an increase in macrophage infiltration in epicardial adipose tissue[13].

It has been suggested that the tissue around the coronary arteries contributes to atherosclerotic plaque development. The association between inflammation of the outer layer of the coronary artery wall, the adventitia, and plaque instability is an established observation[14]. Macrophage density and lymphocytes in the adventitia are associated with plaque instability[15;16]. Also inflammation, determined by macrophage infiltration, of the adipose tissue surrounding the coronary arteries was observed to be associated with adventitia inflammation (**chapter 3**) and plaque instability (**chapter 3, 5**).

In obesity or hypoxic conditions, adipocytes in visceral and subcutaneous adipose tissue are enlarged and will secrete more free fatty acids and cytokines[10;17]. The increased secretion of chemotactic cytokines will lead to increased infiltration of immune cells, mainly macrophages[13;18]. As mentioned before, this is referred to as adipose tissue dysfunction. Adipose tissue dysfunction however, seems to manifest itself in a different way in perivascular adipose tissue. Adipocyte size is comparable in perivascular adipose tissue (pvAT) close to coronary artery segments that are affected with and relatively spared from atherosclerosis (**chapter 3, 4**). These relatively small adipocytes may represent new adipocytes. Small adipocytes together with a reduction of necrotic adipocytes, were observed in a murine model after 20 weeks of high fat diet feeding[19]. The relatively unaffected adipocyte size in pvAT near instable plaques (**chapter 3, 4**) can thus be explained by turnover of dysfunctional adipocytes by new adipocytes. However, despite of their small size, perivascular pre-adipocytes have a reduced capacity for adipocyte differentiation than pre-adipocytes from subcutaneous adipose tissue[20].

Adipose tissue dysfunction as measured by adipocyte size is not related to atherosclerosis of the adjacent vascular wall. But what is the role of macrophages in pvAT? Macrophages were attracted to the aortic pvAT in a murine model of obsity[21]. Moreover, pvAT macrophage infiltration is more prominent near advanced atherosclerotic plaques, as determined by plaque size and characteristics of plaque instability (**chapter 3**). Macrophages in pvAT can therefore be appointed as important players in the atherosclerotic plaque development.

Perivascular adipose tissue: culprit or protector?

Absence of pvAT has been shown to be detrimental for the vascular tonus[22-24]. In rat aortic and mesenteric rings the vessel tonus is more easily increased in absence of pvAT[22-24]. PvAT consists of brown adipose tissue in rats and may not be comparable to human pvAT. The beneficiary effect of pvAT was confirmed in human white adipose tissue[22].

In addition to being involved in the vascular tonus, pvAT may influence the artery wall by paracrine signaling (**chapter 2**). In a model of absence of pvAT, such as in coronary arteries covered by a myocardial bridge, there is reduction of atherosclerotic changes expressed as a reduction in atherosclerotic lesion progression[25] or calcium score (**chapter 6**). Calcium score is a surrogate measure of atherosclerotic burden[26]. In presence of a myocardial bridge, the coronary arteries are protected from paracrine signaling by pvAT, but also enhancement of laminar flow is suggested as the reason for the observation that the coronary artery segment is relatively spared from atherosclerosis[27].

Cross talk between adipocytes and macrophages by paracrine signaling is inducing a local pro-inflammatory environment[10]. In general, macrophages residing in adipose tissue are predominantly of the alternatively activated M2 macrophage type[28;29]. In obese states in mice, a phenotypic switch to the pro-inflammatory M1 macrophage type is observed[28]. M1 macrophages are induced by lipopolysaccharide (lps) or interferon-γ, whereas M2 macrophages are induced by IL-4 and IL-13[30]. Observations in murine and in vitro models have led to the conclusions that M1 macrophages are pro-inflammatory and M2 macrophages anti-inflammatory macrophages[28;30;31]. In humans, in in vivo situations, this seems to be an oversimplification. Macrophages expressing the M2 marker CD206 are able to produce pro-inflammatory cytokines[32]. Furthermore there are macrophages that express both CD206 and an M1 marker (CD11c) (**chapter 5**)[33].

There was a predominance of M2 macrophages in coronary pvAT of subjects with coronary artery disease (**chapter 4, 5**). Furthermore, secretion of soluble adhesion molecules such as soluble vascular cell adhesion molecule (sVCAM) and soluble intercellular adhesion molecule (sICAM), and IL-6 was associated with the amount of M2 macrophages in pvAT (**chapter 4**). The predominance of M2 macrophages is most likely reflecting a suppressive modulation of the inflammatory response, because M2 macrophages mainly have an anti-inflammatory secretion pattern featuring prominent IL-10 secretion[30;31]. However, IL-5 and adiponectin secretion, but not IL-10 secretion was associated with M2 macrophage infiltration in pvAT (**chapter 4**). Furthermore, IL-10 production is relatively low in serum free media in comparison to media containing fetal calf serum34. Therefore IL-10 production may not be observed in the optimal conditions in **chapter 4**.

A profound difference in the secretion of pro- and anti-inflammatory adipocytokines was found between stenosis and control pvAT (**chapter 4**). This is not reflected by the macrophage populations residing in pvAT as there was no increase in M2 macrophage infiltration in control pvAT compared to pvAT near stenosis. In patients undergoing CABG, M1 and M2 macrophages were present in equal levels in pvAT near stenotic as in pvAT near non-stenotic coronary artery segments of the same patient (**chapter 4**). Macrophage colony stimulating factor (M-CSF) is involved in dampening of the immune response by stimulating polarization towards a macrophage phenotype with IL-10 secretion (M2 macrophage)[35;36]. M-CSF secretion, however, was not increased in pvAT near non-stenotic as compared to pvAT near non-stenotic coronary artery segments (**chapter 4**). Also a difference in secretion of IL-33, a protein promoting polarization towards an M2-phenotype[37], could not be demonstrated between stenosis and control pvAT. Comparable secretion of proteins

associated with M2 polarization is consistent with the observations of lack of a demonstrably significant difference in M2 macrophage infiltration in pvAT near stenotic and non-stenotic coronary artery segments.

Perivascular adipose tissue and plaque progression: inside to outside or outside to inside signaling?

Instead of pvAT influencing atherosclerotic plaque development, as has been hypothesized by several research groups (**chapter 2**), it may well be possible that it is the other way around: plaque inflammation might as well contribute to inflammation of the surrounding tissues. Macrophage emigration out of the atherosclerotic plaques through the draining lymph vessels is thought to contribute to stabilization of atherosclerotic plaques[38], whereas macrophage retention is one of the hallmarks of plaque instability[39;40]. Netrin-1, a molecule of importance in axonal guidance, is involved in maintenance of inflammation in atherosclerosis by inhibiting emigration of macrophages from atherosclerotic plaques[41]. In future research production of netrin-1 by pvAT macrophages should be investigated to define its role. Is it a regulator of leukocyte emigration from plaques or is it a chemotactic protein active at the site of inflammation?

Desensitization of the immune response in tissue near advanced atherosclerosis may play a role in the unexpected observation that secretion of cytokines involved in the nuclear factor κB (NF-κB) pathway are secreted at higher amounts in pvAT near non-stenotic than in pvAT near stenotic coronary arteries (**chapter 4**). Instead of near non-stenotic coronary artery segments, increased secretion of pro-inflammatory cytokines was expected in pvAT near stenotic coronary artery segments compared to non-stenotic coronary artery segments. Desensitization of the immune response is supported by a clear down-regulation of the toll like receptor (TLR)-NF-κB pathway after vascular injury in vascular surgery reflected by a reduction in TNF-α production in whole blood[42]. Furthermore, it might be that components needed for TLR-activation are lacking in pvAT of subjects with advanced CAD42.

In case of an acute coronary syndrome, not only the myocardium, but also coronary pvAT will be devoid of oxygen. In ischemia, adipose tissue dysfunction will be manifest as the increased secretion of chemokines and other pro-inflammatory cytokines[43]. Endoplasmatic reticulum stress in adipocytes, induced by glucose deprivation and exposure to lipids is thought to be the underlying mechanism of insulin resistance and adipocyte death in obesity[9;44]. Tissue necrosis, adipocyte death induced by ischemia, attracts macrophages[45]. Free lipid droplets, of death adipocytes draw macrophages to the affected adipose tissue, which arrange around the dead adipocyte, forming crown-like structures[46]. Although both CD11c expressing M1 and CD206 expressing M2 macrophages are attracted to dead adipocytes, expression of markers characteristic of M1 macrophages such as interferon γ and nitric oxide synthase are downregulated[45]. This is contradictory, but consistent with lower cytokine secretion in pvAT near stenotic than near non-stenotic coronary artery segments (**chapter 4**). Because macrophages are attracted to the intima after application of cytokines to the outer vascular wall[47;48], it can be both adipose tissue dysfunction or ischemia induced by coronary insufficiency that fuels the fire.

PART 2

Cardiovascular disease: is it insulin resistance or the components or the metabolic syndrome that causes it?

The vascular consequences of the metabolic syndrome have been extensively studied in the general population[49-51]. The metabolic syndrome, characterized as the combination of abdominal obesity, high blood pressure, low plasma HDL, elevated plasma triglycerides and an increased fasting glucose[52], increases the risk of cardiovascular events[49-51].

As pathologic mechanisms of the individual metabolic syndrome components in the induction of cardiovascular disease are overlapping, the combined effect of the metabolic syndrome components should be less than the individual effects according to critics of the metabolic syndrome[53]. However in patients with vascular diseases this was not confirmed. The risk of new cardiovascular events attributed to the metabolic syndrome was higher than the combined risk of the individual components of the metabolic syndrome[54]. Only an increase in waist circumference above 102cm as in the NCEP III definition of the metabolic syndrome[52;55] was associated with an increase in cardiovascular events[54]. The influence of the metabolic syndrome on cardiovascular events may therefore be driven by factors associated with the metabolic syndrome, but not represented by the other components (high triglycerides, low HDL-cholesterol, high fasting plasma glucose, high blood pressure).

Insulin resistance as a consequence of excess visceral obesity has been proposed as a precursor for both type 2 diabetes and atherosclerosis[56]. This is a revisited theory based on the common-soil-hypothesis posed by Michael Stern in the previous century[57]. In a population of patients with manifest arterial disease, insulin resistance alone was shown to be accompanied by an increased incidence of cardiovascular events (**chapter 7**)[58;59]. The association between insulin resistance, measured as homeostasis model of insulin resistance (HOMA-IR), was independent of the individual components of the metabolic syndrome and hsCRP (**chapter 7**). The independent relation between insulin resistance and subsequent cardiovascular events, but not for all individual components would imply that, at least in patients with manifest arterial disease, the clustering of risk factors is of importance in atherosclerotic disease. Moreover, in patients without clinically manifest arterial disease, the risk attributed to presence of the metabolic syndrome and being in the highest quartile of HOMA-IR was equal[60].

Determinants of the relation between CRP and type 2 diabetes

A combination of reduced sensitivity to insulin in the peripheral tissues and insufficient insulin secretion is recognized to be the mechanism underlying the development of type 2 diabetes[61;62]. Dysfunctional adipose tissue is responsible for the insulin resistance observed in obesity as adipo-cytokines, such as TNF-α and IL-6, are secreted in higher amounts in obesity than in the lean state[63]. Moreover the high secretion of free fatty acids observed in insulin resistant adipose tissue stimulates the TLR-receptor, leading to increased secretion of NF-ϰB associated cytokines[64]. The family of insulin receptor substrates (IRS), IRS-1 and IRS-2 in particular are important components in the cascade involved in insulin signalling. Adipo-cytokines, including TNF-α and IL-6, are able to reduce the activity of IRS-1[65;66].

Increases in CRP as a marker of low grade inflammation are associated with an increased risk of cardiovascular incidents[67] and with insulin resistance[68]. Importantly the risk of type 2 diabetes was partly attributed to increased plasma levels of CRP in the general population[69;70] and in subjects with

manifest arterial disease (**chapter 8**). The pathophysiological mechanism linking CRP directly to insulin resistance, and thereby to type 2 diabetes, is through the c-Jun N-terminal kinase (JNK) pathway. CRP is able to stimulate JNK inducing phosphorylation of IRS-1 in vitro[71].

One subject develops type 2 diabetes due to statin use for every three subjects protected from cardiovascular events[72]. This was concluded from a meta-analysis following two previous meta-analyses observing a slight increase in the risk of type 2 diabetes in the combined data of the placebo controlled trials[73;74]. In contrast with the previous results, the incidence rate of type 2 diabetes is equal in patients with manifest arterial disease with and without statins (**chapter 8**). Although the direct cause and effect are difficult to determine in observational studies, we can conclude that in patients with manifest arterial disease subjects using statins are at comparable risk of developing type 2 diabetes as subjects not on statin therapy. Confounding by indication may play a role in these observations. Statins are prescribed to those at high risk for cardiovascular disease. However, because atherosclerosis and type 2 diabetes share many risk factors such as age and obesity[56], confounding by indication would only exaggerate increased risk of type 2 diabetes due to statin use. The absence of a difference in risk for type 2 diabetes may be due to the fact that subjects with manifest arterial disease are already at high risk to develop type 2 diabetes[56;75] and that statins do not increase the high baseline risk.

The slight increase in risk of type 2 diabetes observed to be attributed to statin use in clinical trials[72-74] is not likely to be caused by an increase in CRP, as the relation between CRP and type 2 diabetes was not modified by statin use (**chapter 8**). The effect of statins on insulin signalling should be protective due to reduction of CRP independent of lipid lowering[76]. CRP is able to induce insulin resistance through the JNK-pathway[71]. The reduction of plasma CRP by statins is hence beneficial for peripheral insulin sensitivity.

Plasma CRP levels are related to obesity, with visceral adipose tissue being more tightly associated than subcutaneous adipose tissue[6]. In multivariate analyses the relation between CRP and insulin resistance (HOMA-IR) was influenced by measures of obesity (**chapter 8**). Insulin resistance together with decreased insulin secretion underlies the development of type 2 diabetes. The attenuation of the CRP attributed increase in insulin resistance after adjustment for visceral adipose tissue quantity confirms that obesity is in the causal pathway of inflammation and type 2 diabetes.

In conclusion, consistent associations between pvAT inflammation and plaque instability were observed, characterized by predominance of anti-inflammatory M2 macrophage infiltration in pvAT. The relation between pvAT macrophages and coronary artery stenosis was not accompanied by increased cytokine secretion, possibly explained by the M2 phenotype of these macrophages. Furthermore insulin resistance and inflammation as systemic results of adipose tissue dysfunction in patients with manifest arterial disease are precursors of cardiovascular events and type 2 diabetes.

REFERENCE LIST

1 Bluher M. The distinction of metabolically 'healthy' from 'unhealthy' obese individuals. Curr Opin Lipidol 2010; 21(1):38-43.

2 Hamer M, Stamatakis E. Metabolically Healthy Obesity and Risk of All-Cause and Cardiovascular Disease Mortality. J Clin Endocrinol Metab 2012.

3 Bonora E, Kiechl S, Willeit J, Oberhollenzer F, Egger G, Targher G et al. Prevalence of insulin resistance in metabolic disorders: the Bruneck Study. Diabetes 1998; 47(10):1643-1649.

4 Ferrannini E, Natali A, Bell P, Cavallo-Perin P, Lalic N, Mingrone G. Insulin resistance and hypersecretion in obesity. European Group for the Study of Insulin Resistance (EGIR). J Clin Invest 1997; 100(5):1166-1173.

5 Sims EA. Are there persons who are obese, but metabolically healthy? Metabolism 2001; 50(12):1499-1504.

6 Faber DR, van der GY, Westerink J, Visseren FL. Increased visceral adipose tissue mass is associated with increased C-reactive protein in patients with manifest vascular diseases. Atherosclerosis 2010; 212(1):274-280.

7 Janssen I, Katzmarzyk PT, Ross R. Waist circumference and not body mass index explains obesity-related health risk. Am J Clin Nutr 2004; 79(3):379-384.

8 Kanhai DA, Kappelle LJ, van der GY, Uiterwaal CS, Visseren FL. The risk of general and abdominal adiposity in the occurrence of new vascular events and mortality in patients with various manifestations of vascular disease. Int J Obes (Lond) 2011.

9 Van Gaal LF, Mertens IL, De Block CE. Mechanisms linking obesity with cardiovascular disease. Nature 2006; 444(7121):875-880.

10 Hajer GR, van Haeften TW, Visseren FL. Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 2008; 29(24):2959-2971.

11 Spencer M, Unal R, Zhu B, Rasouli N, McGehee RE, Jr., Peterson CA et al. Adipose Tissue Extracellular Matrix and Vascular Abnormalities in Obesity and Insulin Resistance. J Clin Endocrinol Metab 2011.

12 Mazurek T, Zhang L, Zalewski A, Mannion JD, Diehl JT, Arafat H et al. Human epicardial adipose tissue is a source of inflammatory mediators. Circulation 2003; 108(20):2460-2466.

13 Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest 2003; 112(12):1796-1808.

14 Maiellaro K, Taylor WR. The role of the adventitia in vascular inflammation. Cardiovasc Res 2007; 75(4):640-648.

15 Moreno PR, Purushothaman KR, Fuster V, O'Connor WN. Intimomedial interface damage and adventitial inflammation is increased beneath disrupted atherosclerosis in the aorta: implications for plaque vulnerability. Circulation 2002; 105(21):2504-2511.

16 Tavora F, Kutys R, Li L, Ripple M, Fowler D, Burke A. Adventitial lymphocytic inflammation in human coronary arteries with intimal atherosclerosis. Cardiovasc Pathol 2010; 19(3):e61-e68.

17 Skurk T, berti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. J Clin Endocrinol Metab 2007; 92(3):1023-1033.

18 Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest 2003; 112(12):1821-1830.

19 Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, DeFuria J, Jick Z et al. Adipocyte death, adipose tissue remodeling, and obesity complications. Diabetes 2007; 56(12):2910-2918.

20 Chatterjee TK, Stoll LL, Denning GM, Harrelson A, Blomkalns AL, Idelman G et al. Proinflammatory phenotype of perivascular adipocytes: influence of high-fat feeding. Circ Res 2009; 104(4):541-549.

21 Police SB, Thatcher SE, Charnigo R, Daugherty A, Cassis LA. Obesity promotes inflammation in periaortic adipose tissue and angiotensin II-induced abdominal aortic aneurysm formation. Arterioscler Thromb Vasc Biol 2009; 29(10):1458-1464.

22 Gao X, Mi S, Zhang F, Gong F, Lai Y, Gao F et al. Association of chemerin mRNA expression in human epicardial adipose tissue with coronary atherosclerosis. Cardiovasc Diabetol 2011; 10:87.

23 Lohn M, Dubrovska G, Lauterbach B, Luft FC, Gollasch M, Sharma AM. Periadventitial fat releases a vascular relaxing factor. FASEB J 2002; 16(9):1057-1063.

24 Verlohren S, Dubrovska G, Tsang SY, Essin K, Luft FC, Huang Y et al. Visceral periadventitial adipose tissue regulates arterial tone of mesenteric arteries. Hypertension 2004; 44(3):271-276.

25 Ishikawa Y, Akasaka Y, Ito K, Akishima Y, Kimura M, Kiguchi H et al. Significance of anatomical properties of myocardial bridge on atherosclerosis evolution in the left anterior descending coronary artery. Atherosclerosis 2006; 186(2):380-389.

26 Haberl R, Becker A, Leber A, Knez A, Becker C, Lang C et al. Correlation of coronary calcification and angiographically documented stenoses in patients with suspected coronary artery disease: results of 1,764 patients. J Am Coll Cardiol 2001; 37(2):451-457.

27 Ishikawa Y, Ishii T, Asuwa N, Masuda S. Absence of atherosclerosis evolution in the coronary arterial segment covered by myocardial tissue in cholesterol-fed rabbits. Virchows Arch 1997; 430(2):163-171.

28 Lumeng CN, Bodzin JL, Saltiel AR. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 2007; 117(1):175-184.

29 Zeyda M, Gollinger K, Kriehuber E, Kiefer FW, Neuhofer A, Stulnig TM. Newly identified adipose tissue macrophage populations in obesity with distinct chemokine and chemokine receptor expression. Int J Obes (Lond) 2010; 34(12):1684-1694.

30 Wolfs IM, Donners MM, de Winther MP. Differentiation factors and cytokines in the atherosclerotic plaque micro-environment as a trigger for macrophage polarisation. Thromb Haemost 2011; 106(5):763-771.

31 Ambarus CA, Krausz S, van EM, Hamann J, Radstake TR, Reedquist KA et al. Systematic validation of specific phenotypic markers for in vitro polarized human macrophages. J Immunol Methods 2012; 375(1-2):196-206.

32 Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G et al. Human adipose tissue macrophages are of an anti-inflammatory phenotype but capable of excessive pro-inflammatory mediator production. Int J Obes (Lond) 2007; 31(9):1420-1428.

33 Zeina AR, Odeh M, Blinder J, Rosenschein U, Barmeir E. Myocardial bridge: evaluation on MDCT. AJR Am J Roentgenol 2007; 188(4):1069-1073.

34 Bzowska M, Nogiec A, Skrzeczynska-Moncznik J, Mickowska B, Guzik K, Pryjma J. Oxidized LDLs Inhibit TLR-induced IL-10 Production by Monocytes: A New Aspect of Pathogen-Accelerated Atherosclerosis. Inflammation 2012.

35 Ambarus CA, Santegoets KC, van BL, Wenink MH, Tak PP, Radstake TR et al. Soluble Immune Complexes Shift the TLR-Induced Cytokine Production of Distinct Polarized Human Macrophage Subsets towards IL-10. PLoS One 2012; 7(4):e35994.

36 Fleetwood AJ, Lawrence T, Hamilton JA, Cook AD. Granulocyte-macrophage colony-stimulating factor (CSF) and macrophage CSF-dependent macrophage phenotypes display differences in cytokine profiles and transcription factor activities: implications for CSF blockade in inflammation. J Immunol 2007; 178(8):5245-5252.

37 Kurowska-Stolarska M, Stolarski B, Kewin P, Murphy G, Corrigan CJ, Ying S et al. IL-33 amplifies the polarization of alternatively activated macrophages that contribute to airway inflammation. J Immunol

2009; 183(10):6469-6477.

38 Bellingan GJ, Caldwell H, Howie SE, Dransfield I, Haslett C. In vivo fate of the inflammatory macrophage during the resolution of inflammation: inflammatory macrophages do not die locally, but emigrate to the draining lymph nodes. J Immunol 1996; 157(6):2577-2585.

39 Libby P, Aikawa M. Stabilization of atherosclerotic plaques: new mechanisms and clinical targets. Nat Med 2002; 8(11):1257-1262.

40 Virmani R, Burke AP, Farb A, Kolodgie FD. Pathology of the vulnerable plaque. J Am Coll Cardiol 2006; 47(8 Suppl):C13-C18.

41 van Gils JM, Derby MC, Fernandes LR, Ramkhelawon B, Ray TD, Rayner KJ et al. The neuroimmune guidance cue netrin-1 promotes atherosclerosis by inhibiting the emigration of macrophages from plaques. Nat Immunol 2012; 13(2):136-143.

42 Versteeg D, Dol E, Hoefer IE, Flier S, Buhre WF, de KD et al. Toll-like receptor 2 and 4 response and expression on monocytes decrease rapidly in patients undergoing arterial surgery and are related to preoperative smoking. Shock 2009; 31(1):21-27.

43 Wood IS, de Heredia FP, Wang B, Trayhurn P. Cellular hypoxia and adipose tissue dysfunction in obesity. Proc Nutr Soc 2009; 68(4):370-377.

44 Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004; 306(5695):457-461.

45 Fischer-Posovszky P, Wang QA, Asterholm IW, Rutkowski JM, Scherer PE. Targeted deletion of adipocytes by apoptosis leads to adipose tissue recruitment of alternatively activated M2 macrophages. Endocrinology 2011; 152(8):3074-3081.

46 Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 2005; 46(11):2347-2355.

47 Miyata K, Shimokawa H, Kandabashi T, Higo T, Morishige K, Eto Y et al. Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. Arterioscler Thromb Vasc Biol 2000; 20(11):2351-2358.

48 Shimokawa H, Ito A, Fukumoto Y, Kadokami T, Nakaike R, Sakata M et al. Chronic treatment with interleukin-1 beta induces coronary intimal lesions and vasospastic responses in pigs in vivo. The role of platelet-derived growth factor. J Clin Invest 1996; 97(3):769-776.

49 Lakka HM, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, Tuomilehto J et al. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. JAMA 2002; 288(21):2709-2716.

50 Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR et al. Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. Circulation 2004; 110(10):1245-1250.

51 Wang J, Ruotsalainen S, Moilanen L, Lepisto P, Laakso M, Kuusisto J. The metabolic syndrome predicts cardiovascular mortality: a 13-year follow-up study in elderly non-diabetic Finns. Eur Heart J 2007; 28(7):857-864.

52 Grundy SM, Cleeman JI, Daniels SR, Donato KA, Eckel RH, Franklin BA et al. Diagnosis and management of the metabolic syndrome: an American Heart Association/National Heart, Lung, and Blood Institute Scientific Statement. Circulation 2005; 112(17):2735-2752.

53 Tenenbaum A, Fisman EZ. "The metabolic syndrome... is dead": these reports are an exaggeration. Cardiovasc Diabetol 2011; 10(1):11.

54 Wassink AM, van der GY, Olijhoek JK, Visseren FL. Metabolic syndrome and the risk of new vascular events and all-cause mortality in patients with coronary artery disease, cerebrovascular disease, peripheral

arterial disease or abdominal aortic aneurysm. Eur Heart J 2008; 29(2):213-223.

55 Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). JAMA 2001; 285(19):2486-2497.

56 Wassink AM, Olijhoek JK, Visseren FL. The metabolic syndrome: metabolic changes with vascular consequences. Eur J Clin Invest 2007; 37(1):8-17.

57 Stern MP. Diabetes and cardiovascular disease. The "common soil" hypothesis. Diabetes 1995; 44(4):369-374.

58 Tenenbaum A, Adler Y, Boyko V, Tenenbaum H, Fisman EZ, Tanne D et al. Insulin resistance is associated with increased risk of major cardiovascular events in patients with preexisting coronary artery disease. Am Heart J 2007; 153(4):559-565.

59 Yanase M, Takatsu F, Tagawa T, Kato T, Arai K, Koyasu M et al. Insulin resistance and fasting hyperinsulinemia are risk factors for new cardiovascular events in patients with prior coronary artery disease and normal glucose tolerance. Circ J 2004; 68(1):47-52.

60 Jeppesen J, Hansen TW, Rasmussen S, Ibsen H, Torp-Pedersen C, Madsbad S. Insulin resistance, the metabolic syndrome, and risk of incident cardiovascular disease: a population-based study. J Am Coll Cardiol 2007; 49(21):2112-2119.

61 Haffner SM, Miettinen H, Gaskill SP, Stern MP. Decreased insulin secretion and increased insulin resistance are independently related to the 7-year risk of NIDDM in Mexican-Americans. Diabetes 1995; 44(12):1386-1391.

62 Stumvoll M, Goldstein BJ, van Haeften TW. Type 2 diabetes: principles of pathogenesis and therapy. Lancet 2005; 365(9467):1333-1346.

63 Pou KM, Massaro JM, Hoffmann U, Vasan RS, Maurovich-Horvat P, Larson MG et al. Visceral and subcutaneous adipose tissue volumes are cross-sectionally related to markers of inflammation and oxidative stress: the Framingham Heart Study. Circulation 2007; 116(11):1234-1241.

64 Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. J Clin Invest 2006; 116(11):3015-3025.

65 Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. Proc Natl Acad Sci U S A 1994; 91(11):4854-4858.

66 Zhong L, Luo Y, Huang C, Liu L. Effect of NF-kappaB decoy on insulin resistance of adipocytes from patients with type 2 diabetes mellitus. Diabetes Metab 2011; 37(6):520-526.

67 Kaptoge S, Di AE, Lowe G, Pepys MB, Thompson SG, Collins R et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. Lancet 2010; 375(9709):132-140.

68 Chao C, Song Y, Cook N, Tseng CH, Manson JE, Eaton C et al. The lack of utility of circulating biomarkers of inflammation and endothelial dysfunction for type 2 diabetes risk prediction among postmenopausal women: the Women's Health Initiative Observational Study. Arch Intern Med 2010; 170(17):1557-1565.

69 Lee CC, Adler AI, Sandhu MS, Sharp SJ, Forouhi NG, Erqou S et al. Association of C-reactive protein with type 2 diabetes: prospective analysis and meta-analysis. Diabetologia 2009; 52(6):1040-1047.

70 Thorand B, Lowel H, Schneider A, Kolb H, Meisinger C, Frohlich M et al. C-reactive protein as a predictor for incident diabetes mellitus among middle-aged men: results from the MONICA Augsburg cohort study, 1984-1998. Arch Intern Med 2003; 163(1):93-99.

71 D'Alessandris C, Lauro R, Presta I, Sesti G. C-reactive protein induces phosphorylation of insulin receptor substrate-1 on Ser307 and Ser 612 in L6 myocytes, thereby impairing the insulin signalling pathway that promotes glucose transport. Diabetologia 2007; 50(4):840-849.

72 Preiss D, Sattar N. Statins and the risk of new-onset diabetes: a review of recent evidence. Curr Opin

Lipidol 2011; 22(6):460-466.

73 Rajpathak SN, Kumbhani DJ, Crandall J, Barzilai N, Alderman M, Ridker PM. Statin therapy and risk of developing type 2 diabetes: a meta-analysis. Diabetes Care 2009; 32(10):1924-1929.

74 Sattar N, Preiss D, Murray HM, Welsh P, Buckley BM, de Craen AJ et al. Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials. Lancet 2010; 375(9716):735-742.

75 Becker A, Bos G, de VF, Kostense PJ, Dekker JM, Nijpels G et al. Cardiovascular events in type 2 diabetes: comparison with nondiabetic individuals without and with prior cardiovascular disease. 10-year follow-up of the Hoorn Study. Eur Heart J 2003; 24(15):1406-1413.

76 Ridker PM, Rifai N, Clearfield M, Downs JR, Weis SE, Miles JS et al. Measurement of C-reactive protein for the targeting of statin therapy in the primary prevention of acute coronary events. N Engl J Med 2001; 344(26):1959-1965.

Chapter 10

Summary

Nederlandse Samenvatting

Dankwoord

Curriculum Vitae

SUMMARY

Rather than being solely a storage depot for triglycerides, adipose tissue is able to secrete pro- and anti-inflammatory cytokines and adipokines. Adipose tissue consists of adipocytes, macrophages and a stromal fraction, all contributing to the secretory function. A state of low grade inflammation, associated with excess adipose tissue, is involved in the increase in the incidences of atherosclerotic diseases and type 2 diabetes that accompany obesity.

The adipose tissue surrounding the arteries, perivascular adipose tissue, is suggested to influence the vascular wall by inducing a local pro-inflammatory environment. In **chapter 2** definitions of adipose tissue depots at different locations are set. In addition, imaging, ex vivo and animal studies are described concerning the relation between perivascular adipose tissue and atherosclerosis.

Cross sections of the left anterior descending artery perivascular were investigated in post mortem cases with atherosclerosis (**chapter 3 and 5**). In **chapter 3** it is reported that the amount of perivascular adipose tissue is related to the size and composition of atherosclerotic plaques. Also inflammation of perivascular adipose tissue, determined by macrophage infiltration is associated with the amount and composition of atherosclerotic plaques. Parameters of plaque composition related to parameters of perivascular adipose tissue were the presence of a lipid core, plaque calcification and macrophage and lymphocyte in filtration in the intima and the adventitia.

A phenotypic switch from the anti-inflammatory, or alternatively activated, macrophage phenotype to the pro-inflammatory, or classically activated, phenotype is observed in adipose tissue in obesity and also in atherosclerotic plaque. However, in perivascular adipose tissue of subjects with coronary atherosclerosis, pro-inflammatory CD11c+ macrophages are sparse compared to anti-inflammatory CD206+ macrophages (**chapter 4 and 5**). In **chapter 5** the relation between macrophage subtypes and atherosclerotic plaque characteristics is studied. Anti-inflammatory CD206+ macrophages in perivascular adipose tissue and the adventitia are related to plaque inflammation and the presence of a lipid core.

In patients undergoing coronary artery bypass grafting, perivascular adipose tissue biopsies were harvested near stenotic and non-stenotic coronary artery segments (**chapter 4**). Surprisingly, multiple adipocytokines such as interleukin 1α and interleukin 5 were secreted at higher levels by perivascular adipose tissue near non-stenotic than near stenotic coronary artery segments. This may reflect a down-regulation of the pro-inflammatory response in advanced atherosclerosis as anti-inflammatory macrophages are more abundant in perivascular adipose tissue of these patients (**chapter 4 and 5**). However, a difference in the number of M2 macrophages in perivascular adipose tissue near stenotic and non-stenotic coronary artery segments could not be demonstrated.

In addition, absence of perivascular adipose tissue at coronary artery segments covered by a myocardial bridge is associated with a lack of atherosclerosis. Hemodynamic factors have been proposed to explain the absence of atherosclerosis in bridged coronary artery segment, but an alternative view is that bridged coronary arteries are protected from the influence of perivascular adipose tissue by the overlying myocardium. The relation between the presence of a myocardial bridge and (a low) atherosclerotic burden was determined on computed tomography-scans in **chapter 6** by measuring the calcium score. The retrieved results were confirmed by determining the presence of atherosclerotic plaques in bridged coronary artery segments. The association between the presence of a myocardial bridge and a low calcium score was attenuated when

adjusting for local perivascular adipose tissue thickness (0mm at bridged coronary artery segments and 0.3mm or more at control coronary artery segments).
Together these results indicate potential involvement of perivascular adipose tissue in atherosclerotic plaque development, although the causality of the relation has yet to be determined.
In obese subjects the peripheral tissues become insulin resistant. Insulin resistance is accompanied by a cluster of metabolic changes, often referred to as the metabolic syndrome. Metabolic syndrome is highly prevalent in patients with manifest arterial disease and is associated with advanced vascular damage, thereby identifying those patients with an even higher cardiovascular risk. In patients with manifest arterial disease without known diabetes, insulin resistance increases with the number of metabolic syndrome components (**chapter 7**). In addition, elevated insulin resistance increases the risk of new cardiovascular events. The relation between insulin resistance and cardiovascular events is independent of the components of the metabolic syndrome.
Obesity-induced inflammation is thought to be a 'common soil' underlying both cardiovascular disease and type 2 diabetes. Dysfunctional adipose tissue produces large amounts of free fatty acids and in addition it is able to secrete several pro-inflammatory cytokines. Subsequent activation of toll-like receptor-4 on adipocytes by free fatty acids further increases the production of pro-inflammatory cytokines and also induces insulin resistance by affecting the intracellular insulin signaling cascade. Plasma high sensitivity C-reactive protein (hsCRP), as a measure of low grade inflammation, is a predictor and a risk factor for cardiovascular events. In **chapter 8** it is shown that patients that already have manifest arterial disease with the highest plasma hsCRP levels were at the highest risk to develop type 2 diabetes. In addition they were more insulin resistant as compared to those with low hsCRP levels. This increase in risk is more pronounced in females than in males and is not modified by statin use.
In conclusion, adipose tissue is able to induce effects leading to atherosclerosis and diabetes on a local and systemic level.

NEDERLANDSE SAMENVATTING

Obesitas, gedefinieerd als een body mass index groter dan 30 kg/m^2, wordt nu, naast dat het geassocieerd is met type 2 diabetes en kanker, erkend als een risicofactor voor hart- en vaatziekten. Bij een groot deel van de mensen met overgewicht gaat toename van de hoeveelheid buikvet gepaard met dysfunctie van vetweefsel. Hiermee wordt bedoeld dat het vetweefsel behalve dat het een opslagfunctie voor vrije vetzuren heeft, ook signalen uitzendt in de vorm van onstekingseiwitten (adipocytokines). Vetweefsel bestaat onder meer uit vetcellen, ontstekingscellen, cellen van kleine bloedvaatjes en voorlopercellen van vetcellen. Zowel vetcellen als ontstekingscellen, met name macrofagen, zijn in staat om ontstekingseiwitten te produceren. Een verhoogde productie van deze ontstekingseiwitten leidt op zijn beurt weer tot de migratie van macrofagen naar vetweefsel. Ten opzichte van slanke mensen worden bij obese mensen ontstekingseiwitten als interleukine 6 en tumor necrosis factor α in verhoogde mate, en het ontstekingsremmende eiwit adiponectine in verminderde mate in het bloed gemeten.

Vet rond vaten wordt ook wel perivasculair vet genoemd. Er wordt gedacht dat dit vet invloed heeft op de vaatwand door het creëren van een pro-inflammatoir (ontstekingsbevorderend) milieu. Ontstekingseiwitten diffunderen door het weefsel naar de vaatwand en zorgen voor het aantrekken van macrofagen en bindweefselcellen in de vaatwand. In tegenstelling tot deze theorie werd voorheen gedacht dat aderverkalking, beter gezegd verbindweefseling van de vaatwand (atherosclerose) alleen veroorzaakt wordt door invloeden vanuit de bloedbaan. Uiteindelijk ontstaat door atherosclerose van de vaatwand een vernauwing van de vaten of een plaque, verdikking, die gevoelig is voor verstopping door bloedstolsels. Het tegengaan van het proces van atherosclerose van buitenaf of binnenuit is daarom belangrijk om hart- en vaatziekten tegen te gaan.

In **hoofdstuk 2** wordt er een overzicht gegeven van de nomenclatuur van de verschillende vetdepots en de literatuur omtrent perivasculair vet in relatie tot atherosclerose. In dierstudies werd gezien dat applicatie van ontstekingseiwitten aan de buitenkant van het bloedvat leidt tot een toename van de hoeveelheid atherosclerose en inflammatie in de aanliggende vaatwand. Verder laten case-control studies zien dat de hoeveelheid vet rond het hart groter is bij patiënten met vaatlijden van de kransslagaderen dan bij mensen zonder vaatlijden van de kransslagaderen. Bovendien is het eiwit-profiel van het vet rond het hart meer pro-inflammatoir bij patiënten met vaatlijden van de kransslagaderen dan bij patiënten met hartkleplijden.

Om te onderzoeken of er een verband is tussen lokale kenmerken van vet en atherosclerose van de aanliggende vaatwand zijn kransslagaderen met het omliggende vet onderzocht. Om zowel het omliggende vet als de kransslagader te onderzoeken zijn er doorsnedes van de kransslagaderen gemaakt van 16 obductiecasus met atherosclerose. Iedere halve centimeter werd er een doorsnede van de linker kransslagader gemaakt en deze werd onder de microscoop onderzocht. In **hoofdstuk 3** wordt beschreven dat er een relatie is tussen de hoeveelheid perivasculair vet en atherosclerose in het aanliggende vat. Hierbij is er zowel naar kwantiteit als kwaliteit van de atherosclerotische plaque gekeken. De kwantiteit van de plaque wordt beschreven als de plaque grootte en de kwaliteit als de compositie van de plaque. Parameters waarnaar gekeken werd bij plaque compositie waren de aanwezigheid van een lipide houdende kern, kalk in de plaque en de hoeveelheid ontstekingscellen in de plaque. Naast de hoeveelheid perivasculair vet waren ook kenmerken van vetontsteking, namelijk de hoeveelheid macrofagen en vetcelgrootte gerelateerd aan de plaquegrootte en de compositie van de plaque in de aanliggende vaatwand. In de analyses werd rekening gehouden met

het feit dat er per persoon meerdere doorsnedes genomen zijn.
In **hoofdstuk 4** is de secretie van ontstekingseiwitten door perivasculair vetweefsel bestudeerd in vetbiopten die afgenomen werden tijdens open hart operaties. De hart-longchirurg nam een biopt af van het vetweefsel vlak naast een vernauwd kransslagadersegment en vlak naast een kransslagadersegment dat geen tekenen van atherosclerose vertoonde op de beelden van de diagnostische hartkatheterisatie. Hoewel van tevoren verwacht werd dat er evenveel of meer pro-inflammatoire eiwitten in het vetweefsel bij vernauwde kransslagader-segmenten dan in het vetweefsel bij niet vernauwde kransslagader-segmenten gemeten zouden worden, werd juist bij niet vernauwde segmenten meer eiwitsecretie geobserveerd. Van de 22 eiwitten die gemeten werden waren er 5 anti- en pro-inflammatoire eiwitten in hogere concentraties gemeten in vetsamples van niet vernauwde kransslagadersegmenten ten opzichte van vernauwde segmenten. Mogelijk dat bij vergevorderde atherosclerotische plaques het immuunsysteem juist minder actief wordt als een beschermingsmechanisme om uitgebreide schade door overmatige activatie te beperken. Macrofagen, ontstekingscellen, werden wel in verhoogde mate gevonden bij vernauwde segmenten. Hoewel het merendeel van de aanwezige macrofagen van het anti-inflammatoire type was in beide samples, was er geen relatie tussen de aanwezigheid van een vernauwing en de hoeveelheid anti-inflammatoire macrofagen.
Ook in de microscopiedoorsnedes van de eerdergenoemde obductiecasus werden vooral anti-inflammatoire macrofagen in het vet rondom de kransslagader geobserveerd. Er werden extra kleuringen van de microscopie doorsnedes gedaan om de verschillende subtypes van macrofagen aan te tonen. In **hoofdstuk 5** wordt beschreven dat anti-inflammatoire macrofagen in perivasculair vet gerelateerd zijn aan de mate van ontsteking van de atherosclerotische plaque van de vaatwand. Ontsteking van de plaque werd gekwantificeerd door de oppervlakte van de totale hoeveelheid macrofagen in de plaque te meten. De toename in pro-inflammatoire macrofagen in perivasculair vet bij toenemende ontsteking van de plaque kon niet statistisch aangetoond worden. Ook de hoeveelheid anti-inflammatoire macrofagen in de buitenste laag van de vaatwand nam toe met toenemende ontsteking van de binnenste laag van de vaatwand, maar voor pro-inflammatoire macrofagen kon dit niet aangetoond worden.
De hypothese dat vet invloed heeft op de vaatwand werd vervolgens getest door middel van een model waarin vetweefsel afwezig is (**hoofdstuk 6**). Dit is het geval bij de anatomische variatie waarbij een kransslagader een deel van zijn traject niet in het vet rondom het hart vervolgt maar door de hartspier. In deze getunnelde kransslagadersegmenten, myocardial bridges, is er geen atherosclerose gemeten. De mate van atherosclerose werd bepaald aan de hand van de lokale calciumscore en de aanwezigheid van atherosclerotische plaques. Uit analyse blijkt dat de afwezigheid van atherosclerose in myocardial bridges deels wordt gedreven door de afwezigheid van vet. Correctie van de analyse voor de dikte van het perivasculaire vet (die was 0 mm bij een myocardial bridge en 0.3 of meer mm bij vaatsegmenten zonder myocardial bridge) maakte namelijk dat het verband verdween.

In **hoofdstuk 7 en 8** ligt de focus op visceraal vet bij mensen met manifest vaatlijden in relatie met atherosclerose en type 2 diabetes. In een cohort van patiënten met klinisch manifest vaatlijden werd de relatie tussen insuline resistentie en een het optreden van een tweede vasculaire manifestatie (hartinfarct, beroerte, vasculaire dood) bestudeerd. Patiënten met insuline resistentie bleken een grotere kans te hebben op het krijgen van een tweede cardiovasculaire manifestatie (**hoofdstuk 7**). Deze relatie was onafhankelijk van de componenten van het metabool syndroom. Componenten van

het metabool syndroom zijn een verhoogde middelomtrek, een hoge bloeddruk, een verhoogd nuchter glucose, verhoogde triglyceriden, en een laag HDL in het bloed. Het metabool syndroom is sterk gerelateerd aan obesitas en insuline resistentie.
Laaggradige ontsteking veroorzaakt door secretie van ontstekingseiwitten is een van de links tussen obesitas en atherosclerose. In **hoofdstuk 8** wordt de relatie tussen laaggradige ontsteking en het ontstaan van type 2 diabetes beschreven. Met het stijgen van het high sensitive CRP als maat voor ontsteking stijgt ook de kans op het krijgen van diabetes. De relatie tussen laaggradige ontsteking en insuline resistentie wordt minder sterk na correctie voor de hoeveelheid buikvet. De relatie werd niet beïnvloed door correctie voor de dikte van het onderhuidse vet, suggererend dat met name het buikvet een pro-inflammatoir milieu veroorzaakt dat leidt tot insuline resistentie en diabetes.
In **hoofdstuk 9** worden de resultaten van de verschillende onderzoekingen in groter verband bekeken en geïnterpreteerd.

Concluderend wordt in dit proefschrift aangetoond dat:

- de hoeveelheid perivasculair vetweefsel gerelateerd is aan de grootte en de compositie van de atherosclerotische plaque in de naburige vaatwand, net als de hoeveelheid macrofagen in het perivasculaire vet.
- de secretie van pro- en anti-inflammatoire eiwitten hoger is in perivasculair vet naast een niet vernauwd kransslagadersegment dan naast een atherosclerotisch kransslagadersegment.
- perivasculair vet met name anti-inflammatoire macrofagen bevat en dat deze anti-inflammatoire macrofagen gerelateerd zijn aan ontsteking van de atherosclerotische plaque en de aanwezigheid van een lipide houdende kern in de plaque..
- de afwezigheid van perivasculair vet, zoals bij kransslagadersegmenten overdekt door een myocardial bridge, gepaard gaat met afwezigheid van atherosclerose in dat segment
- bij patiënten met manifest vaatlijden insulineresistentie het risico op een tweede vasculair event verhoogt en dat dit risico onafhankelijk is van de componenten van het metabool syndroom
- inflammatie gerelateerd is aan insuline resistentie en diabetes bij patiënten met manifest vaatlijden en dat deze relatie met name verklaard kan worden door een toename van de hoeveelheid buikvet.

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Als ik iets heb geleerd in de afgelopen paar jaar is dat ik niet alles alleen kan en dat het ook nergens voor nodig is om dat te willen kunnen. De totstandkoming van een goed onderzoeksprotocol, net zoals de uitvoering ervan, heeft de visie van meerdere mensen en ook tijd nodig. Ik ben iedereen die mij bij de verschillende onderzoeken die in dit proefschrift beschreven staan, hebben bijgestaan dan ook zeer dankbaar. Waarschijnlijk ben ik toch nog veel mensen vergeten met naam en toenaam te noemen. Bij voorbaat mijn excuses hiervoor.

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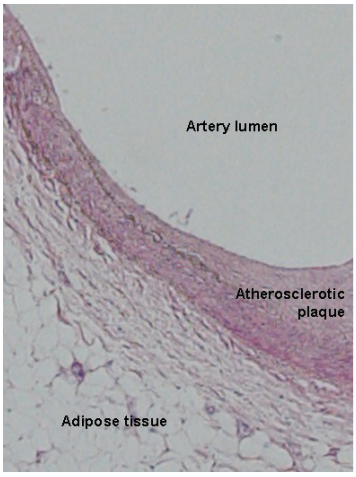
CURRICULUM VITAE

Sandra Verhagen was born on November 20th or 22nd (officially registered date of birth is November 20th 1982) in Kandy, Sri lanka. She graduated high school in 2001 from the Liemers College in Zevenaar. In 2002 she started medical school and became a medical doctor in 2008 after which she started her work as a PhD student in the University Medical Center Utrecht.
Under the supervision of F.L.J. Visseren (department of Vascular Medicine) and Y. van der Graaf (department of Epidemiology, Julius Center) she initiated the studies described in the this thesis. In May 2012 she started her training in Internal Medicine in the Gelderse Vallei Hospital in Ede under the supervision of dr. R. Heijligenberg.

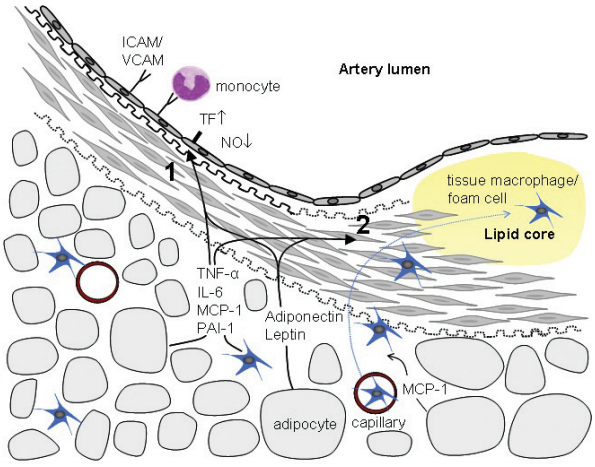
Color Appendix

Chapter 2; page 17

Figure 1 | Perivascular adipose tissue and its possible involvement in atherogenesis



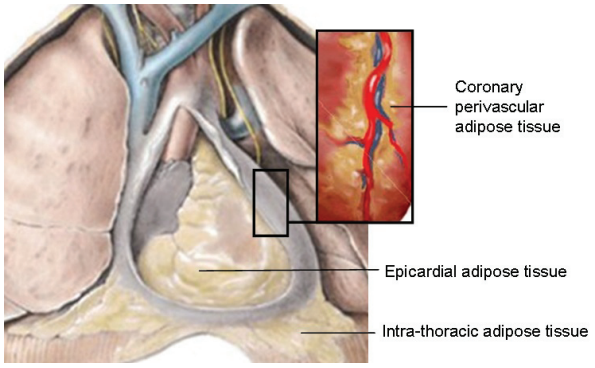
A. Histologic reproduction of the coronary artery and surrounding tissue. Perivascular adipose tissue is in close association with the artery wall which enables diffusion of adipokines and cytokines produced by the perivascular adipose tissue.



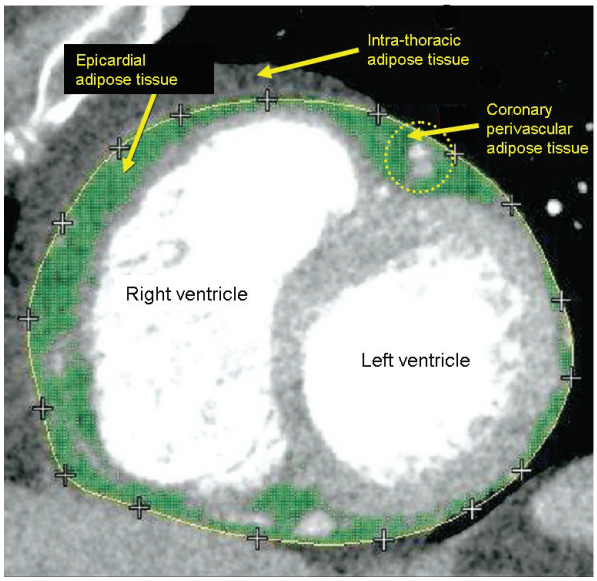
B. Model of the artery wall and perivascular adipose tissue. Adipocytes enlarge and increase the production of pro-inflammatory cytokines and adipokines. Cytokines such as TNF-alpha and IL-6 are also produced by macrophages in adipose tissue. Pro-inflammatory products of adipose tissue are able to diffuse to the surrounding tissues and structures.
(1) Paracrine effects of cytokines and adipokines may be exerted on the endothelium causing endothelial dysfunction (decreased NO production), hypercoagulability (tissue factor and PAI-1 are upregulated), increased chemotaxis by upregulated MCP-1 and adhesion of monocytes to the endothelium by increased expression of adhesion molecules. (2) Also paracrine effects may be excerted directly on the adjacent tissues leading to influx of tissue macrophages into the artery wall from 'outside to inside' and to proliferation of smooth muscle cells.

Chapter 2; page 18

Figure 2 | Nomenclature and quantification of adipose tissue within the mediastinum

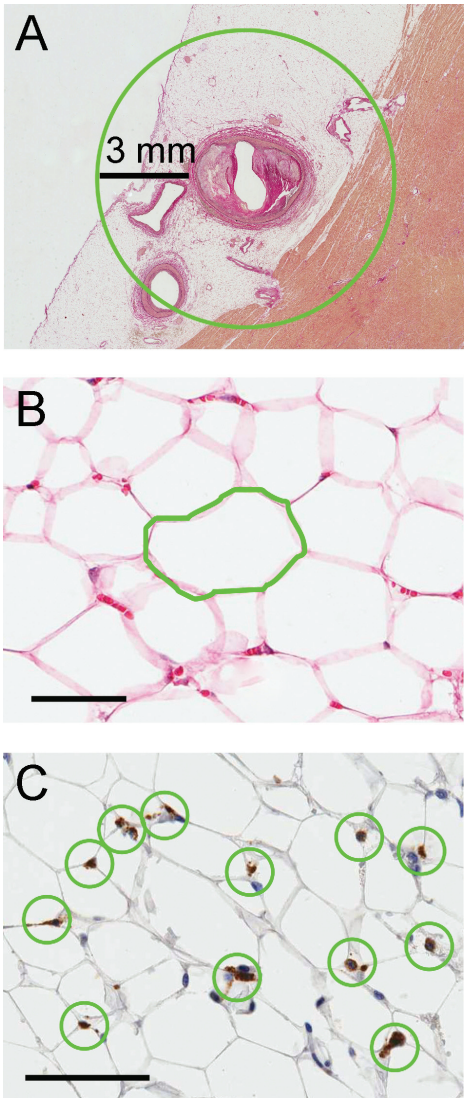


A. Epicardial adipose tissue is defined as the adipose tissue within the pericardium. Intra-thoracic adipose tissue is defined as the adipose tissue within the mediastinum, but outside the pericardial sac. Perivascular adipose tissue is adipose tissue around arteries irrespective of location. Coronary perivascular adipose tissue is defined as the adipose tissue directly surrounding the coronary arteries.



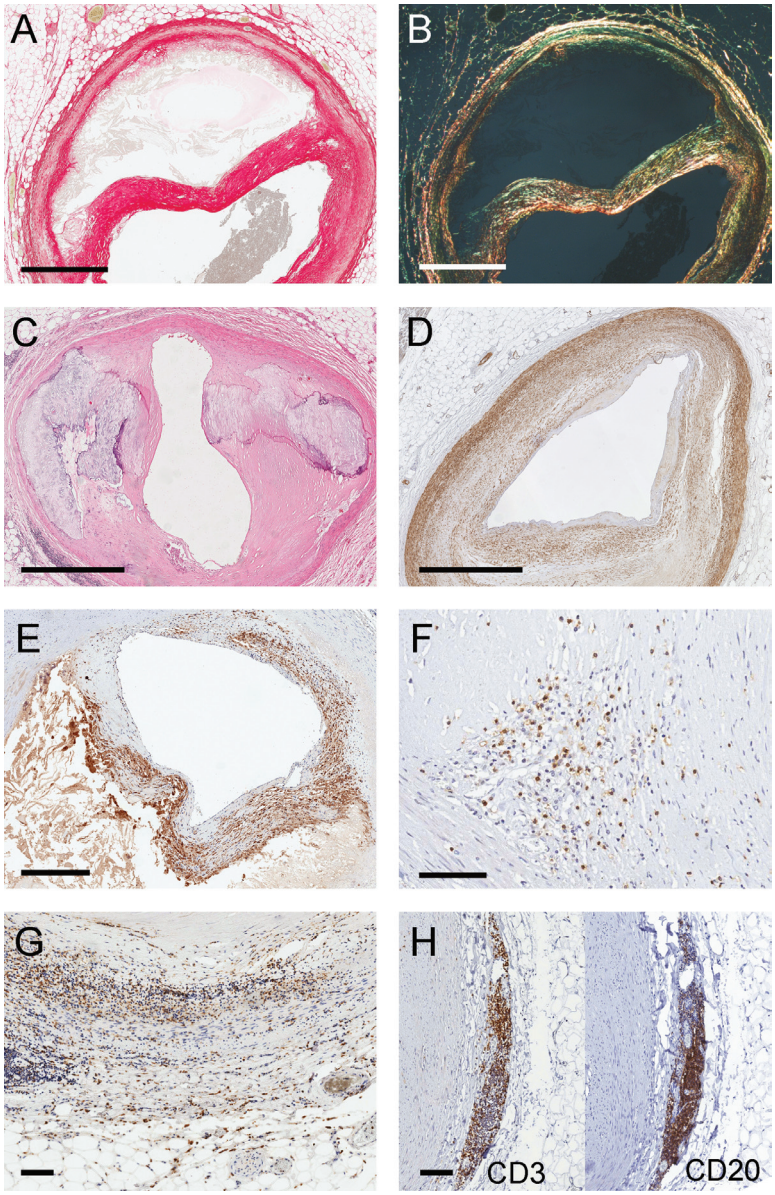
B. Quantification of epicardial adipose tissue on CT image: Axial computed tomography image adapted from Gorter et al. Am. J. Cardiol. 2008[31]. Epicardial adipose tissue is measured by manually tracing the pericardium. Within the traced region, containing the heart and the epicardial adipose tissue, the adipose tissue is isolated by selecting a density range.

Chapter 3; page 42



Supplemental figure 1 | Methods
A: Measurement of pvAT(%): the percentage of adipose tissue area within the area of the circle (radius of 3 mm outside of the adventitia); B, measurement of adipocyte area Bar = 50 µm; C, pvAT macrophages Bar = 100 µm.

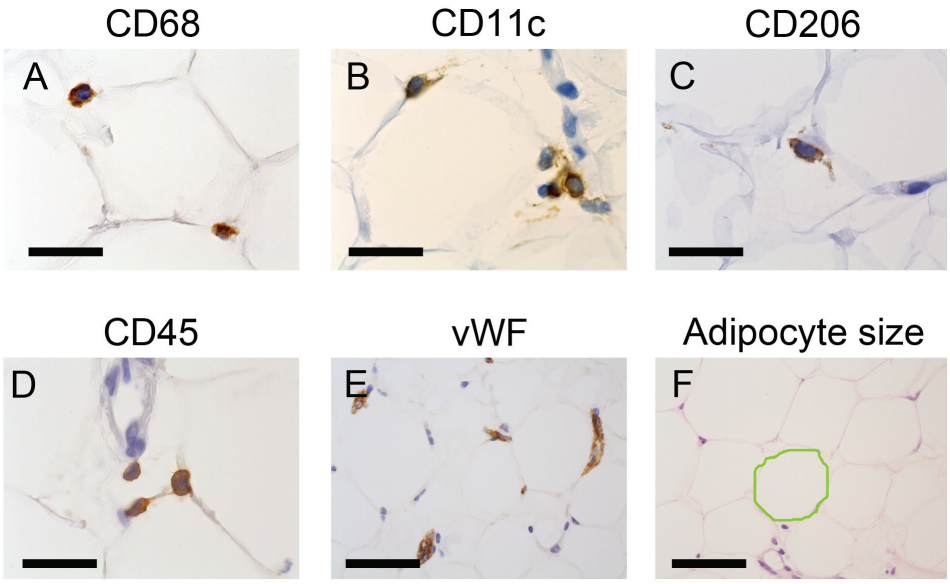
Chapter 3; page 43



Supplemental figure 2 | Histology of plaque and adventitia.
A, Picrosirius red staining showing a lipid core (>40% of plaque area: category 3); collagen content (<50% of plaque area). Bar = 1mm. B, Picrosirius red staining with polarized light. Bar = 1mm. C, H&E-staining of a plaque with calcification (10-40% of plaque area: category 2). Bar = 1mm. D, αSMA staining with smooth muscle cells (> 50% of plaque area). E, CD68 staining with groups of >50 macrophages in the plaque (category 3). Bar = 500 μm. F, CD3 staining showing a group of >25 lymphocytes in the plaque shoulder (category 3). Bar = 100 μm. G, CD68 staining showing >25 adventitia macrophages (category 3). The upper side of photograph: plaque, at the bottom: adventitia; Bar = 100 μm. H, CD3 and CD20 stainings of the adventitia showing an aggregate of >100 lymphocytes in the adventitia (category 3) consisting of T- and B-cells. Bar = 100 μm.

Chapter 4; page 53

Figure 1 | Histological characteristics of perivascular adipose tissue (n=38).



a | Immunohistochemistry stainings
A: CD68 positive macrophages; B: M1 macrophages, CD11c positive; C: M2 macrophages, CD206 positive; D: lymphocytes, CD45 positive; E: endothelial cells, Factor 8/ von Willebrand factor (vWF) positive; F: adipocyte size indicated by the area within the green circumference. Bars indicate 20µm (A to D) or 100µm (E and F).

Chapter 5; page 67

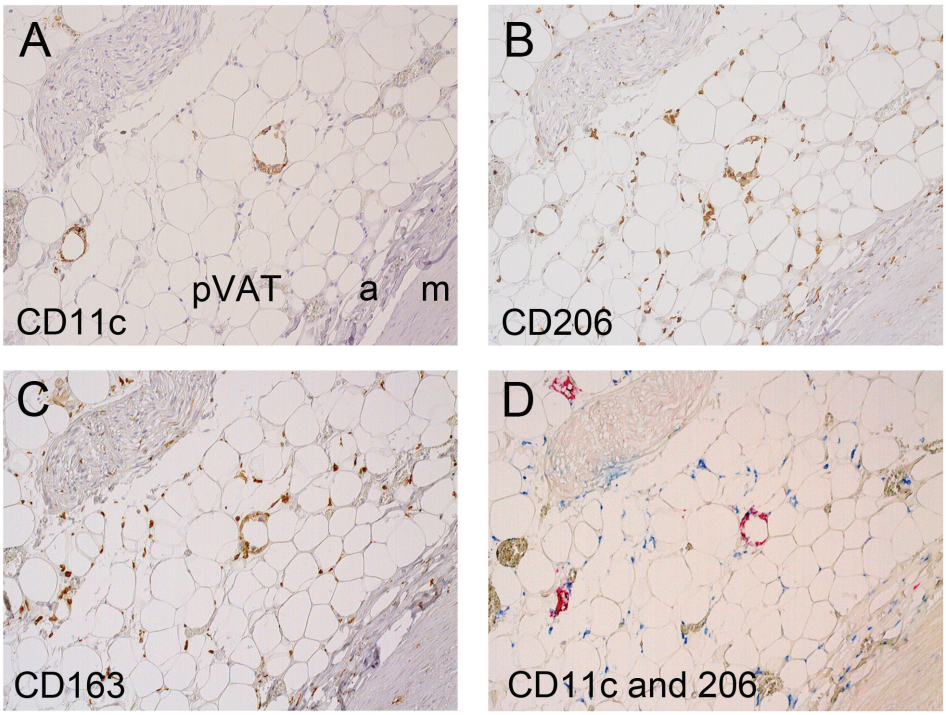


Figure 2 | Immuno-histochemistry stainings of macrophage subtypes
Immuno-histochemistry stainings of M1 and M2 macrophages in coronary perivascular adipose tissue (pVAT). Consecutive sections: A: CD11c immuno-histochemistry staining showing some positive cells (in brown) in pVAT. m, media; a, adventitia (magnification 100x). B and C: CD206 and CD163 immuno-histochemistry stainings showing abundant staining (in brown) in pVAT and the adventitia. D: double immuno-histochemistry staining for CD11c (in red) and CD206 (in blue).

Chapter 6; page 78

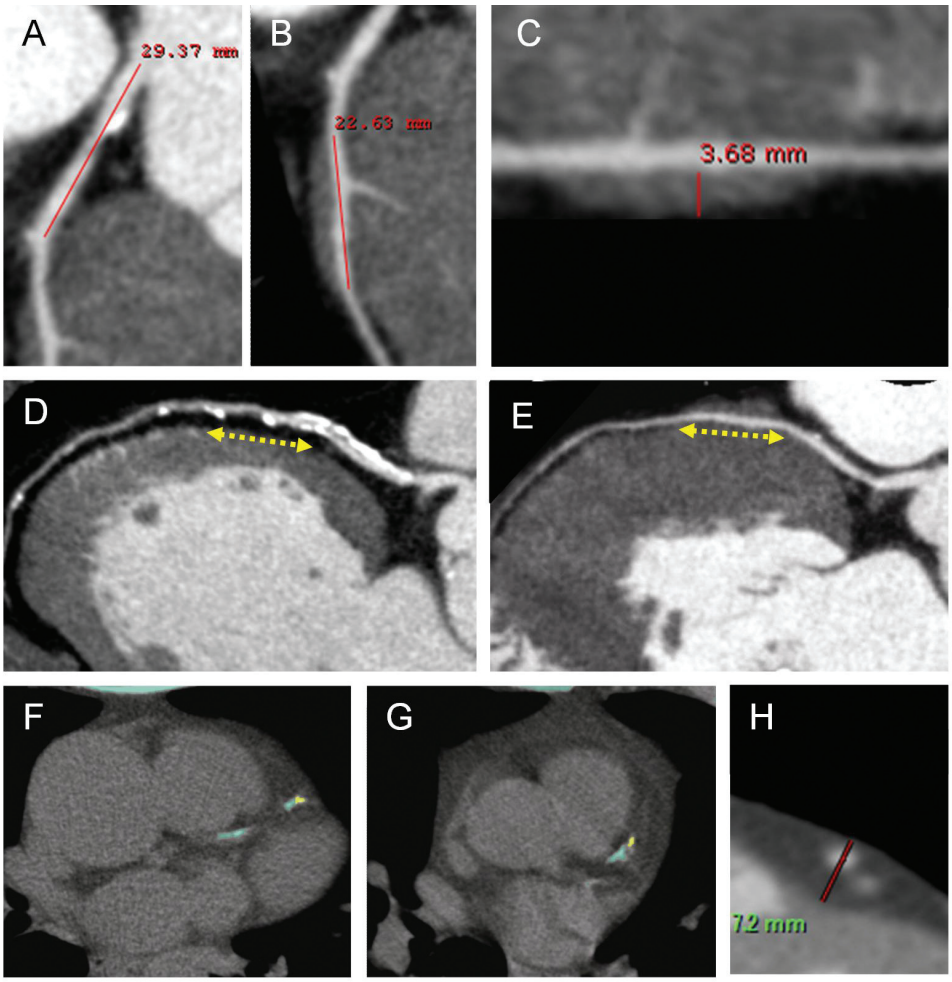


Figure 1 | Detection of myocardial bridges (MBs), measurement of MB length and thickness, and measurement of calcium score of coronary artery segments with and without an MB.
The following MB properties were measured: distance to origin (A), length (B), and thickness (C). Calcium score was measured in bridged coronary artery segments and corresponding segments in subjects without an MB. In subjects without an MB (D), calcium score was measured in a segment at the same distance to origin and over the same length as in subjects with an MB (E). (Selected regions are indicated with a yellow arrow). Calcium score was measured in the region of interest (F) (G). (Blue: regions with a density of >130 Houndsfield Units (HU). (Yellow: selected region). Perivascular adipose tissue thickness was measured in the regions of interest (H).