Postprandial dysmetabolism and cell-derived microparticles as cardiovascular risk factors in metabolic syndrome and type 2 diabetes mellitus

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The studies presented in this thesis were performed at the Diabetes Center of the Department of Internal Medicine, VU University Medical Center, Amsterdam in collaboration with the Department of Clinical Chemistry, Academic Medical Center, Amsterdam, The Netherlands, and at the Department of Internal Medicine, Hospital Amstelland in Amstelveen, The Netherlands. Parts of this work were supported by the Dutch Diabetes Research Foundation (grant no. 2000.025).

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Postprandial dysmetabolism and cardiovascular disease in type 2 diabetes

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ABSTRACT

The worldwide prevalence of type 2 diabetes mellitus has reached epidemic proportions. The so-called traditional risk factors cannot fully explain the excessive cardiovascular disease (CVD) risk of type 2 diabetic patients. Numerous studies indicate that postprandial metabolic derangements, most notably hyperglycemia and hypertriglyceridemia, which are exaggerated and prolonged in type 2 diabetes, are important CVD risk factors since they induce oxidative stress and endothelial dysfunctions. Here we review the current evidence showing that postprandial dysmetabolism may indeed constitute an important CVD risk factor as well as the mechanisms underlying this association. Finally, some possible therapeutic options and recommendations for future research are discussed.

INTRODUCTION

The worldwide prevalence of type 2 diabetes mellitus is growing rapidly, reaching epidemic proportions.[1] One of the major reasons of the increased prevalence in developing countries, is the adoption of the so-called Western lifestyle, i.e. a high intake of energy dense food and a low physical activity pattern. These life-style changes lead to one of the key abnormalities underlying type 2 diabetes mellitus, i.e. insulin resistance. Insulin resistance is associated with central obesity, hyperinsulinemia, polycystic ovary syndrome, hypertension and dyslipidemia.[2,3] Hyperglycemia, the established diagnostic marker of diabetes mellitus, is the result of the second key feature, progressive pancreatic beta-cell failure.

It is well recognized that type 2 diabetic patients have an excess risk of developing atherosclerosis, resulting in high cardiovascular disease (CVD) morbidity and mortality.[4] Therefore, with the rise of the prevalence of diabetes, it may be expected that the global burden of CVD will also increase. Since the so-called traditional risk factors, such as high cholesterol, hypertension, smoking, and low HDL-cholesterol, cannot fully explain the excessive CVD risk of type 2 diabetic patients, other risk factors need to be identified.[5]

Two important processes involved in the development of atherosclerosis, the underlying cause of CVD, are inflammation and coagulation activation.[6] Although the exact mechanisms underlying atherogenesis are still not completely understood, vascular endothelial dysfunction is generally believed to be the starting point.[6,7] Under normal conditions, the multiple functional characteristics of the endothelium, including regulation of the vascular tone, thrombogenesis, vascular wall permeability, and cell growth, collectively protect the vascular system.[7] Adequate production of nitric oxide (NO) plays a pivotal role in the majority of these processes. Since endothelial functions cannot be measured directly, several indirect methods have been developed to estimate these functions. These include the assessment of plasma concentrations of endothelial-cell derived proteins, including Van Willebrand factor (vWF) and vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1),[7] as well as the more complicated measurement of NO-dependent vasodilatation or flow-mediated dilatation (FMD) of the brachial artery by non-invasive ultrasound.[8] Increased plasma concentrations of vWF, VCAM-1 and ICAM-1, and a decreased FMD have been associated with an increased risk of CVD.[7,8]

Other CVD risk factors which have been recognized recently include hyperhomocysteinemia, elevated plasma levels of C-reactive protein (CRP), reflecting a low-grade inflammatory state, fibrinogen, representing a hypercoagulable state, and oxidized-low density

lipoprotein (ox-LDL), as an indicator of oxidative stress.[9,10] However, only recently, the excessive and prolonged metabolic disturbances occurring in the postprandial state in type 2 diabetic subjects, have regained interest as potential CVD risk factors.[11]

Already over 200 years ago, William Heberden made the first observation regarding a postprandial effect on the circulation of blood,[12] and in the late seventies of the previous century, Zilversmit postulated that atherosclerosis is a postprandial phenomenon.[13] Ever since, a large body of evidence has accumulated indicating a relation between postprandial dysmetabolism, especially hyperglycemia and hypertriglyceridemia, and the risk of CVD.[11,14-17]

In this review we will briefly discuss the present evidence for postprandial dysmetabolism as a potential CVD risk factor, with special emphasis on postprandial glucose and lipid dysmetabolism. To this purpose, the association of postprandial dysmetabolism with the presence of (indicators of) atherosclerotic vascular disease and the possible underlying mechanisms will be reviewed.

Postprandial hyperglycemia

Various mechanisms keep the plasma glucose levels in healthy subjects between strict limits, even after a carbohydrate load. The insulin response following a meal regulates the glucose uptake from the blood into the peripheral tissues and inhibits gluconeogenesis and glycogenolysis in the liver. Beta-cell dysfunction (e.g. loss of the normal insulin secretion pattern) and insulin resistance contribute to glucose intolerance and both can be found very early in the disease process, finally leading to type 2 diabetes.[2]

In the United Kingdom Prospective Diabetes Study (UKPDS), lowering of glycated hemoglobin (HbA_{1c}) mainly reduced long term microvascular complications of diabetes, whereas effects on macrovascular disease were less convincing.[18] In apparent contrast, several epidemiological studies have shown an association between 2-hour glucose concentrations following a 75 g glucose load (2hPG) and the occurrence of CVD in the general population.[19,20] A meta-analysis of 20 studies with more than 95 000 people demonstrated a continuous relationship between postload glucose levels and CVD risk extending into the non-diabetic range.[19] The DECODE study demonstrated that the 2hPG concentrations, even in subjects with normal fasting glucose, was associated with mortality, independent of fasting plasma glucose concentrations.[20]

Chronic hyperglycemia has been associated with impaired endothelial function.[21] Recent studies in healthy and type 2 diabetic subjects indicate that acute hyperglycemia causes endothelial dysfunction as measured by FMD.[22] Also, circulating ICAM-1 plasma levels

significantly increased in both diabetic and normal subjects after an oral glucose tolerance test, suggesting endothelial cell activation.[23]

Ceriello and co-workers have shown that postprandial hyperglycemia is accompanied by several alterations of the coagulation system.[24,25] An oral glucose load in both healthy and type 2 diabetes patients caused a shortening of the half-life of fibrinogen and an increase in plasma fibrinopeptide A and the fragments of prothrombin and factor VII. In addition, acute, short-term hyperglycemia resulted in a transient hyper-reactivity of platelets to high shear stress, combined with a significant rise of plasma vWF in patients with type 2 diabetes.[26] Taken together, these findings suggest that hyperglycemia may induce a hypercoagulable state.

In healthy and impaired glucose tolerance (IGT) subjects, consecutive pulses of intravenous glucose increased circulating cytokine concentrations (interleukin 6 (IL-6) and tumor necrosis factor α (TNFα)) to a greater extent than during similar blood glucose levels which were kept stable during a hyperglycemic clamp. This effect was more pronounced in subjects with IGT.[27] The same investigators showed changes in IL-6 (but not TNFα) plasma concentrations in type 2 diabetic patients after a carbohydrate meal.[28] Thus, blood glucose excursions may induce a pro-inflammatory response.

Numerous *in vitro* studies demonstrated cytotoxic effects of high glucose levels in various cell types.[29-31] Of interest is the demonstration by Risso and colleagues that intermittent high glucose levels induced more apoptosis than constant corresponding glucose levels in human umbilical vein endothelial cells.[30]

Four main molecular mechanisms underlying the hyperglycemia-induced vascular damage have recently been reviewed,[31] all of which are the result of intracellular hyperglycemia. These include increased polyol pathway influx; increased advanced glycation end-product (AGE) formation; activation of protein kinase C (PKC) isoforms; and increased hexosamine pathway flux. These seemingly different mechanisms are the result of a single process, i.e. overproduction of superoxide by the mitochondrial electron-transport chain. This hyperglycemia-induced oxidative stress ultimately results in modification of intracellular proteins resulting in an altered function, DNA damage, activation of the transcription factor NF-κB, causing abnormal changes in gene expression, decreased production of NO, and increased expression of cytokines, growth factors and pro-coagulant and pro-inflammatory molecules.[31]

Taken together, postload or postprandial glucose levels are associated with enhanced risk of CVD. However, most epidemiological studies addressing the contribution of postload glucose levels to CVD risk, especially the early ones, did not take into account

the earlier mentioned classical risk factors, such as dyslipidemia. In studies investigating the relationship between postload glucose and CVD risk, adjustment for blood pressure, lipids and smoking, resulted in considerable attenuation of this association.[32,33] These data indicate that postload glucose may not be an independent CVD risk factor but rather a risk marker, suggestive of underlying other metabolic disturbances, such as insulin resistance and dyslipidemia, that may have an even greater impact on CVD risk.

In conclusion, evidence of postprandial hyperglycemia as an independent risk factor is not convincing. Therefore, the observed association between postload glucose excursions with CVD is at least partly explained by the presence of insulin resistance and related CVD risk factors.

Postprandial hypertriglyceridemia

In the Western diet, more than 40 percent of the energy intake is derived from fats. Figure 1a.1 demonstrates a schematic representation of the metabolic pathways of dietary fats leading to triglyceride-rich lipoproteins (TRL) and endogenous TRL production.[34] In the insulin-resistant state the production of very-low density lipoprotein (VLDL) by the liver is inappropriately high. Together with a reduced lipoprotein lipase (LPL) activity this results, in high triglyceride (TG) concentrations, especially in the postprandial state. The large amount of TRLs and their prolonged residence time in the circulation may lead to increased exchange of the core lipid cholesteryl ester for TG between TRL and LDL and HDL particles mediated by cholesteryl-ester transfer protein (CETP). This process enriches LDL and HDL with TG, and these particles are subsequently more readily hydrolyzed by hepatic lipase resulting in smaller, denser LDL particles and lower concentrations of HDL. These abnormalities may explain the characteristic diabetic dyslipidemia, which is now recognized to be very atherogenic.[34]

Already in 1959 an association between plasma TG concentrations and incident coronary heart disease was reported.[35] However, the known inverse association between TG and HDL cholesterol makes it difficult to show an independent association between plasma TG and atherosclerotic vascular disease. A recently performed meta-analysis including data of 57 000 subjects from 17 studies demonstrated that fasting TG concentrations were an independent risk factor for CVD, also when adjusted for HDL cholesterol.[36] A 1 mmol/l increase in plasma TG was associated with a relative risk of 1.3 for men and 1.8 for women.

In general practice, serum lipid concentrations including TG are measured in the morning after an overnight fast. However, the fasting value should be considered the nadir

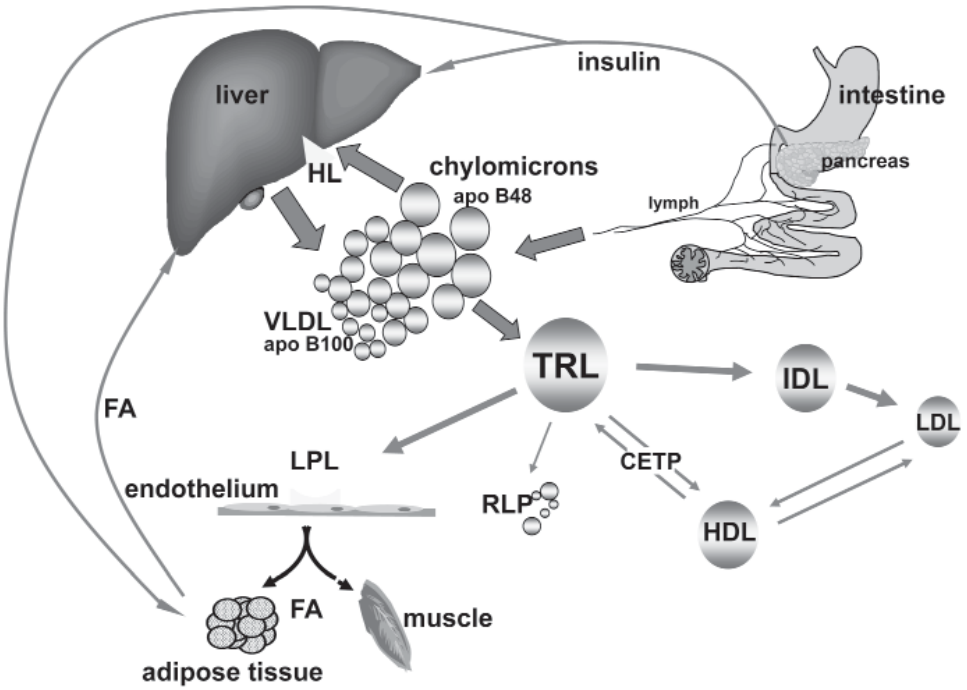

Figure 1a.1 Lipid metabolism *in vivo*. Dietary fatty acids (FA) are absorbed from the gut and converted to triglycerides (TG) to be incorporated into chylomicrons in the intestinal epithelial cells. The TG-rich apoB48 containing chylomicrons enter the plasma via the intestinal lymph. Lipoprotein lipase (LPL) hydrolyses the TG in chylomicrons to FA, which are taken up by muscle cells for oxidation or adipocytes for storage. The remaining particles, the chylomicron remnants, are removed from the circulation by the liver through binding of their surface apo E to the LDL receptor or LDL-receptor-related protein. Very low density lipoprotein (VLDL) particles are TG-rich apo B100 containing particles, synthesized by the liver. As with chylomicrons, VLDL TG are hydrolyzed by LPL. VLDL-remnants or intermediate-density lipoproteins (IDL) are taken up by liver receptors via apoE or converted to low-density lipoproteins (LDL). Chylomicrons, VLDL and their respective remnants (RLP, remnant lipoproteins) are termed TG-rich lipoproteins (TRL). Under physiological conditions, insulin, which is elevated in the postprandial state, suppresses lipolysis from adipose tissue and hepatic VLDL production, however, this insulin action is inappropriate in insulin-resistance and type 2 diabetes, resulting in high TRL concentrations. The large amount of TRL and their prolonged residence time in the circulation increase the exchange of esterified cholesterol from HDL and LDL to TRL and of TG to LDL and HDL particles, which is mediated by cholesterol-ester transfer protein (CETP). TG-enrichment of LDL particles renders them better substrates for hepatic lipase (HL), which hydrolyses TG from the core of LDL and turns them into smaller and denser particles. Small dense LDL are more atherogenic as they readily enter the subendothelial space and become oxidized (ox-LDL). TG-enriched HDL particles are smaller and are more rapidly catabolized, which may explain the observed low plasma HDL in insulin resistance and type 2 diabetes.[36]

of the 24-h TG profile and could therefore be misleadingly low. In the past few years several clinical studies have suggested that high postprandial TRL may be related to coronary heart and/or carotid artery disease in non-diabetic and diabetic subjects.[37-39] The Physician Health study, including 14 916 men aged 40 to 84 years, with a follow up of 7 years, showed that the non-fasting TG concentrations strongly predicted incident myocardial infarctions, with a RR of 1.40 (95% CI, 1.10-1.77) per 1.13 mmol/L increase.[38] This study suggests that random or postprandial TG concentrations, are an important indicator of CVD risk. Although fasting TG levels are the most important determinant of postprandial TG levels,[40] it may be argued that in insulin resistant subjects with a delayed postprandial TRL clearance, non-fasting TG should be used to approximate overall TG exposure.

In male patients following a myocardial infarction, Karpe and co-workers found that the progression of coronary lesions over 5 years was related to the postprandial plasma levels of small chylomicron remnants (Sf 20-60 apolipoprotein B-48).[41] Adjustment for the possible confounding effect of HDL and dense LDL apolipoprotein B concentrations did not substantially alter the strength of this association. In line with these findings are coronary angiography data described by Mero et al., which suggest that especially small chylomicron remnants are implicated in the progression of coronary artery disease.[16]

In healthy and type 2 diabetic subjects, endothelial dysfunction, measured by ultrasound as FMD, has been associated with high TG excursions.[42,43] Correlations between postprandial TRL, impaired FMD and oxidative stress markers have been demonstrated, suggesting that free radical production may be an underlying mechanism.[42,43] Supportive of this hypothesis is the finding that in healthy subjects this effect could be attenuated by the antioxidant vitamin C.[43]

In vitro studies demonstrated increased adhesion molecule expression in endothelial cells after incubating with chylomicrons and VLDL.[44,45] Using rat arterial rings, Lundman et al, showed impairment of endothelium-dependent relaxation following exposure to the TG-containing fat emulsion Intralipid, however, exposure to VLDL did not affect vascular function.[45]

Postprandial coagulation activation by TRL was demonstrated by several investigators, however the underlying mechanism(s) are not fully understood.[46,47] An elegant study performed by Silveira et al. suggests an important role for the intrinsic coagulation pathway, based on *in vivo* activation of factor XI by TG.[46] Other prothrombotic changes occurring with an oral fat load are increased PAI-1 activity and PAI-1 antigen.[48] Postprandial lipemia enhanced platelet P-selectin expression without affecting other markers of platelet activation.[49]

The effect of a high fat meal (50 g of fat) on cytokine concentrations, reflecting the inflammatory state, was studied in healthy and type 2 diabetic patients.[28] In healthy subjects, significant correlations were found between postprandial TG and TNFα levels, whereas in diabetic patients also a positive correlation between postprandial plasma TG and IL-6 concentrations was observed. Anti-oxidant supplementation lowered the rise of the cytokines, suggesting that the cytokine response to TG was mediated by oxidative stress.

To summarize, historically, in diabetic patients, most emphasis was laid on hyperglycemia, whereas recent evidence demonstrate the importance of dyslipidemia, in particular hypertriglyceridemia, as a CVD risk factor. Although at present, epidemiological and long-term intervention studies are largely lacking, *in vivo* data convincingly show an association between postprandial TRL and indicators of CVD. Similar to postprandial hyperglycemia, both *in vivo* and *in vitro* studies indicate that (postprandial) TG elevations are proinflammatory, prothrombotic, and adversely affect several endothelial functions, by inducing oxidative stress (Figure 1a.2). Therefore, it is feasible, that prolonged postprandial hypertriglyceridemia leads to an atherogenic environment *in vivo*. However, as for postprandial hyperglycemia the evidence for postprandial hypertriglyceridemia as independent in cardiovascular disease is still scanty. More evidence, which can only be obtained from large prospective studies, is certainly required.

Therapeutical interventions

Drugs that have been effective in reducing meal-related glucose excursions are the α-glucosidase inhibitors, the short acting insulin analogues and the meglitinides.[50-52]

Recently, the STOP-NIDDM study showed a lower incidence of hypertension and myocardial infarction after treatment with the α-glucosidase inhibitor acarbose.[53] These findings should be interpreted with some caution as the study was not designed to assess the effect of acarbose on CVD end-points. The prospectively predefined end-point was conversion to diabetes, however, in the final report ten different CVD end-points were mentioned, including angina and peripheral vascular disease. In addition, it can not be excluded that the reduction in CVD end-points is the result of lowering TG, since a TG lowering effect of acarbose is described.[54]

Metformin is the only blood glucose-lowering drug that has been shown to lower diabetes-related and CVD endpoints in obese type 2 diabetic patients. Based on the proposed working mechanism,[55] an effect on meal-related glucose excursions can not be expected, but metformin was shown to reduce postprandial chylomicron concentrations.[56]

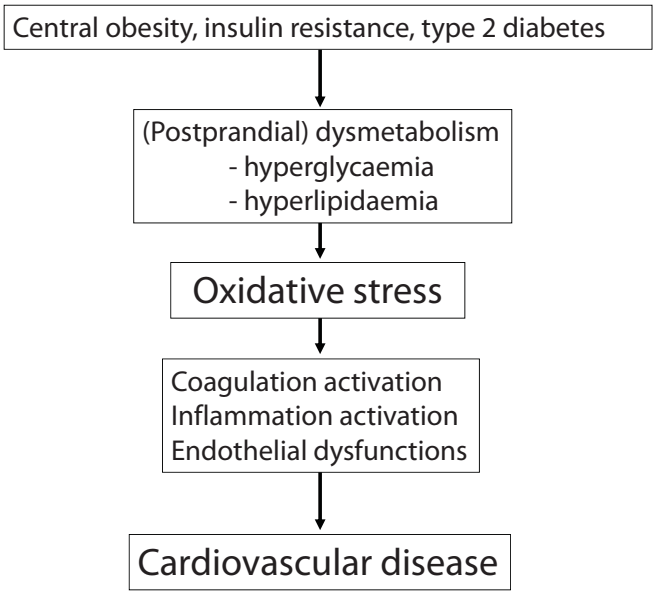

Figure 1a.2 Insulin resistant states and type 2 diabetes are characterized by high circulating levels of atherogenic lipid particles due to an increased supply of fatty acids to the liver and defective hepatic clearance of lipoproteins. In the postprandial state, the lipid abnormalities are further exaggerated, with an additional adverse effect of meal-induced hyperglycaemia. These postprandial metabolic derangements increase the production of reactive oxygen species causing oxidative stress and functional abnormalities of the vascular endothelium at several levels, including impairment of vasoreactivity, increased coagulation and inflammation activation and increased vascular permeability. Collectively, postprandial dysmetabolism and the associated oxidative stress may link insulin resistance and type 2 diabetes to the disproportional incidence of CVD in these high-risk populations.

Correction of postprandial hyperglycemia, for example with insulin secretion enhancers, will not only affect glucose levels but probably also the postprandial lipid responses. However, recently the group of Taskinen demonstrated that nateglinide and glibenclamide increased postprandial insulin secretion and decreased postprandial glycemia, but neither drug attenuated postprandial lipemia in type 2 diabetic subjects with good glycemic control.[57]

Intensive insulin treatment may improve diabetic dyslipidemia to some extent, however, an entire correction of the atherogenic lipid profile, including postprandial hypertriglyceridemia, may not be achieved.[58]

The lipid lowering drugs, i.e. hydroxymethylglutarylco-enzyme A (HMG-CoA) reductase inhibitors (statins), and peroxisome proliferator-activated receptor (PPAR) alpha agonists (fibrates), are established as most efficient agents that reduce CVD morbidity and mortality in various high-risk populations.[59,60] Statins inhibit cholesterol synthesis and up-regulate the hepatic LDL receptor whereas fibrates increase LPL activity and limit hepatic VLDL secretion. Based on their respective working mechanisms, the most benefit on postprandial lipidemia may be expected from fibrates. Indeed, fibrate treatment reduced postprandial TG levels by 30-50%.[61] The proposed beneficial effect of fibrates on postprandial endothelial function measured by FMD, however, is disappointing.[61,62] Statins tend to induce a modest lowering of both fasting and postprandial TG.[61,63] However, statin treatment showed a marked beneficial effect on postprandial induced oxidative stress and endothelial function.[63] Independently of their cholesterol-lowering action, statins seem to have anti-inflammatory and vasculoprotective effects ('pleiotropic' effects).[64]

We conclude that the high CVD morbidity and mortality associated with type 2 diabetes is at least partly due to a prolonged and exaggerated postprandial state in these patients. To date, however, controlled randomized intervention studies, showing that postprandial glucose- and TG lowering results in amelioration of clinically relevant endpoints are lacking.

These conclusions should in no way distract from the therapeutic aim to achieve target HbA1c and lipid values in patients with type 2 diabetes.

Recommendations for future research and testing postload dysmetabolism

As discussed above, although the beneficial effect of therapy targeting postprandial dysmetabolism still needs to be established, studies assessing the true atherogenic exposure of the vascular system in high-risk patients should abandon the classical glucose-centered view and use physiological tests combining glucose and lipid loads. Most earlier-mentioned studies demonstrated the effects of postprandial dysmetabolism on a single and rather artificial challenge, like a liquid 75 g glucose or liquid fat load. In daily life, most meals consumed are mixed and of solid consistence. Ceriello and co-workers showed a cumulative adverse effect of postprandial hypertriglyceridemia and hyperglycemia on endothelial function.[63] The effect of a single component challenge possibly underestimates the real-life postprandial dysmetabolic state and we therefore recommend the use of standardized mixed-meal containing of at least 75 g of carbohydrates and 50 g of fat in future postprandial (intervention) studies.

ABBREVIATIONS

2hPG: 2-hour glucose concentrations following a 75 g glucose load; AGE: Advanced glycation end-product; CETP: Cholesteryl-ester transfer protein; CRP: C- reactive protein; CVD: Cardiovascular disease; DECODE: Diabetes Epidemiology : Collaborative analysis Of Diagnostic criteria in Europe; FMD: Flow mediated dilatation; HbA1c: glycated hemoglobin; HDL: High density lipoprotein; HMG-CoA: Hydroxymethylglutarylco-enzyme A; ICAM-1: intercellular adhesion molecule-1; IGT: Impaired glucose tolerance; IL-6: Interleukin-6; LPL: lipoprotein lipase; NF-κB: Nuclear factor-κB; NO: Nitric oxide; ox-LDL: Oxidized-low density lipoprotein; PAI-1: Plasmin activator inhibitor-1; PKC: Protein kinase C; PPAR: Peroxisome proliferator-activated receptor; TG: Triglyceride; TNFα: Tumor necrosis factor α; TRL: Triglyceride rich lipoprotein; UKPDS: United Kingdom Prospective Diabetes Study VCAM-1: Vascular cellular adhesion molecule-1; VLDL: Very low density lipoprotein; vWF: Van Willebrand factor.

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1b

Cellular microparticles: new players in the field of vascular disease?

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ABSTRACT

Microparticles are small membrane vesicles that are released from cells upon activation or during apoptosis. Cellular microparticles in body fluids constitute a heterogeneous population, differing in cellular origin, numbers, size, antigenic composition and functional properties. Microparticles support coagulation by exposure of negatively charged phospholipids and sometimes tissue factor, the initiator of coagulation *in vivo*. Microparticles may transfer bioactive molecules to other cells or microparticles, thereby stimulating cells to produce cytokines, cell adhesion molecules, growth factors and tissue factor, and modulate endothelial functions. Microparticles derived from various cells, most notably platelets but also leukocytes, lymphocytes, erythrocytes and endothelial cells, are present in the circulation of healthy subjects. Rare hereditary syndromes with disturbances in membrane vesiculation leading to decreased numbers of microparticles clinically present with a bleeding tendency. In contrast, elevated numbers of microparticles are encountered in patients with a great variety of diseases with vascular involvement and hypercoagulability, including disseminated intravascular coagulation, acute coronary syndromes, peripheral arterial disease, diabetes mellitus and systemic inflammatory disease. Finally, microparticles are a major component of human atherosclerotic plaques.

In view of their functional properties, cell-derived microparticles may be an important intermediate in the cascade of cellular and plasmatic dysfunctions underlying the process of atherogenesis.

INTRODUCTION

Already in the 1940s it was known that human plasma and serum contained a subcellular factor that facilitated fibrin formation.[1,2] It was not until 1967 when, using electron microscopic techniques, Wolf demonstrated that this subcellular factor consisted of small vesicles ("microparticles"), which were called "platelet dust". These microparticles, showed procoagulant activity, comparable to that of intact platelets.[3] Their procoagulant activity was designated as platelet factor 3 (PF3).[4] Subsequently, it was shown that (platelet-derived) microparticles (PMP) were formed during the attachment of platelets to the vascular wall *in vitro*.[5] In recent years, the interest for microparticles has substantially increased, not only because of their procoagulant properties but also because of their putative role in inflammatory processes and their ability to directly affect endothelial functions.[6-9] Their suspected involvement in clinical disease was demonstrated for the first time in patients with idiopathic thrombocytopenic purpura (ITP).[10]

The majority of *in vivo* microparticles in blood is derived from platelets,[11] whereas microparticles from erythrocytes, granulocytes, monocytes, lymphocytes and endothelial cells usually circulate at lower numbers. Interestingly, significant differences exist between microparticle fractions or subpopulations found in the circulation of healthy subjects[12,13] and those found in patients suffering from various diseases with increased thromboembolic risk or vascular damage, such as atherosclerotic vascular disease, sepsis, diabetes mellitus, severe hypertension and end-stage renal failure.[14-22] Also, microparticles constitute an important component of the human atherosclerotic plaque.[23]

To summarize, microparticles are closely associated with the presence and the possible development of atherosclerotic and inflammatory vascular damage. In this review, we describe the structure, detection, pathogenesis and characteristics of microparticles. Finally, the possible clinical relevance of microparticles will be discussed in the context of various diseases.

Characterisation of microparticles: size and composition

Platelet activation plays a key role in the development of arterial thrombosis resulting in major clinical syndromes, such as acute myocardial infarction. During platelets activation, vesiculation of parts of the plasma membrane occurs leading to the formation of PMP, the size of which typically ranges from 0.1 to 1.0 μm. Platelets and other cells are surrounded by a plasma membrane that consists of a phospholipid bilayer, including phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) en sphingomyeline (SM). In unstimulated cells, the distribution of these phospholipids within the bilayer is

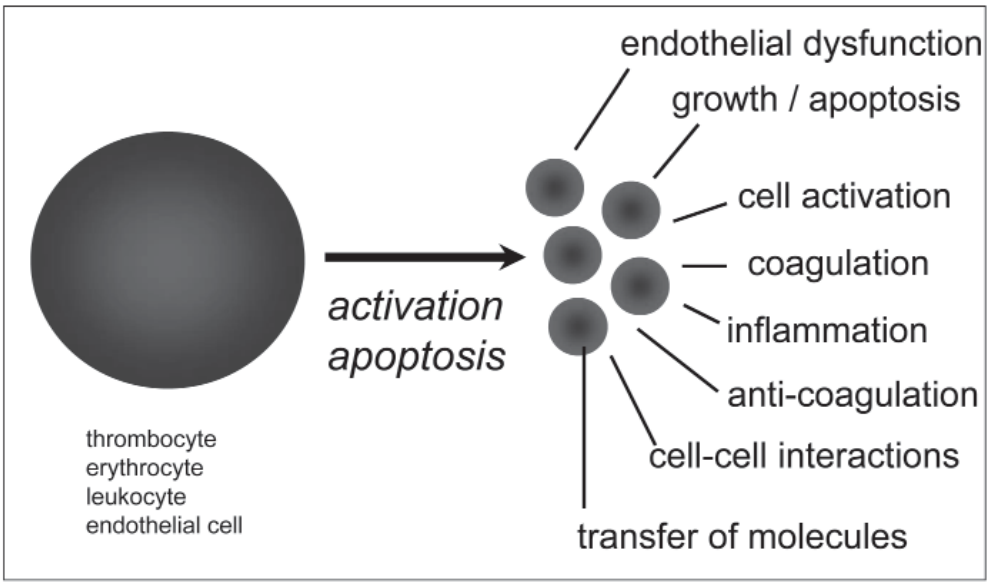

Figure 1b.1 The cellular origin, structure and functional properties of microparticles.

asymmetrical. The neutral (uncharged) phospholipids PC and SM are primarily located in the outer (exoplasmic) membrane leaflet, while the negatively charged PS and PE are present within the inner (cytoplasmic) leaflet. The asymmetrical distribution of phospholipids in the plasma membrane is actively maintained by various enzymes, such as the aminophospholipid-translocase or flippase.[24] During cell activation or apoptosis, the asymmetrical distribution of these phospholipids disappears. As a consequence, negatively charged phospholipids such as PS and PE become (surface) exposed. The intracellular mechanisms underlying the release of microparticles are as yet not fully understood, but they seem to be associated – among others – with the inducing stimulus leading to the actual vesiculation. It is now becoming apparent, that the formation of microparticles is a highly regulated process: the phospholipid composition of PMP shows characteristics from intracellular rather than from plasma membrane fractions and recent studies in endothelial cells showed that constitutively exposed proteins from these cells are hardly transmitted to endothelial cell-derived microparticles.[25,26]

Microparticles expose various antigens, notably those also exposed by their "parent"cells, i.e. the cells from which they are released. For instance, PMP expose glycoproteins GPIb (CD42b), platelet-endothelium adhesion molecule-1 (PECAM-1; CD31) and the fibrinogen receptor, the integrin αIIbβ3 (GPIIb-IIIa). In addition, PMP can expose activation markers

such as P-selectin (CD62P). Similarly, microparticles from other cells can be characterized: examples are microparticles from erythrocytes that stain for glycophorine A, granulocytic microparticles for CD66, monocytic microparticles for CD14, lymphocytic microparticles for CD4 and CD8 and endothelial cell-derived microparticles for CD31, CD34, CD51 (vitronectin), CD62E, and CD146 (MUC18, S-Endo-1).[13,14,17-22,26] All these microparticles can also expose activation markers that are characteristic of their respective "parent"cell.

Detection of microparticles

Flowcytometry

Microparticles can be detected by flow cytometry in blood samples or fractions there from, as well as in other body fluids such as synovial fluid.[6,27] Using labelled antibodies against 1) cell-specific antigens and/or activation markers and 2) annexin V, a protein that binds specifically to negatively charged phospholipids in the presence of calcium ions, microparticle fractions or subpopulations can be quantified and concurrently their cellular origin as well as their "activation status" can be established. To correct for autofluorescence and binding of antibodies to Fc-receptors, microparticles are also stained with a (labelled) control antibody plus annexin V, but without calcium ions. Of each event detected by the flowcytometer, the size (forward scatter, FSC) and density (sidescatter, SSC) are determined electronically, as well as the fluorescence in various channels. Fluorescence reflects the amount of antibody bound and therefore is an estimate for the amount of antigen exposed on the membrane surface. Figure 1b.2 illustrates the visualization of PMP by flow cytometry.

Electron microscopy

Figure 1b.3 shows scanning electron microscopy images of unstimulated cultured human umbilical vein endothelial cells (HUVECS) and the formation of microparticles upon stimulation with interleukin-1α. The diameter of the vesicles released by stimulated HUVECS ranges from 0.1 to 1.0 μm.

Enzyme-linked immunosorbent assay (ELISA)

One of the most frequently used ELISAs to quantify cell-derived microparticles employs a plate coated with annexin V.[7,28,29] Upon addition of a (plasma) sample, microparticles present within this sample will bind to annexin V. After washing, a cell-specific antibody can be added to quantify numbers of cell-specific microparticles. Alternatively, after washing the procoagulant activity of the (bound) microparticles can be determined using a prothrombinase assay.

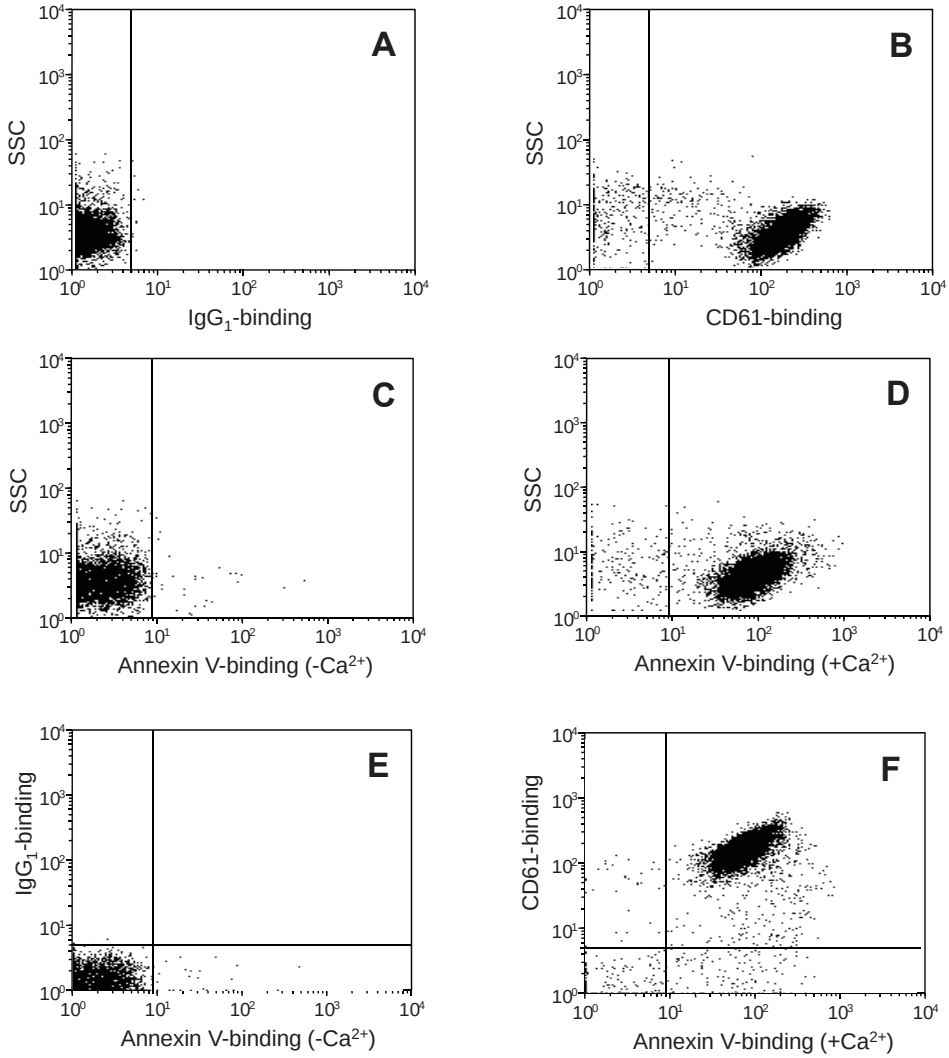

Figure 1b.2 Use of flow cytometry for microparticle analysis. Flowcytometric analysis of whole blood by size (forward scatter, FSC) and density (side scatter, SSC) predominantly yields erythrocytes (visible in the upper right of panel A); Events staining positive for (labelled) antibody directed against platelet-antigen are platelets in R(egion) 2 (B), whereas R3 contains larger events such as complexes of platelets or platelet-derived microparticles, and R1 contains events smaller than platelets, the PMP. In cell-free plasma, microparticles can be analysed after additional centrifugation (C-H). In panel D, the microparticles are stained with an anti-GPIIIa (CD61) monoclonal antibody, as compared to a control antibody (C). Almost all events bind annexin V in the presence of calcium ions (F), but not in the absence of such ions (E). Double staining of microparticles with anti-CD61 plus annexin V in the presence of calcium ions allows visualization of PMP exposing negatively charged phospholipids (H). As a control, microparticles are stained with annexin V in the absence of calcium ions and control IgG_1 antibody (G; control).

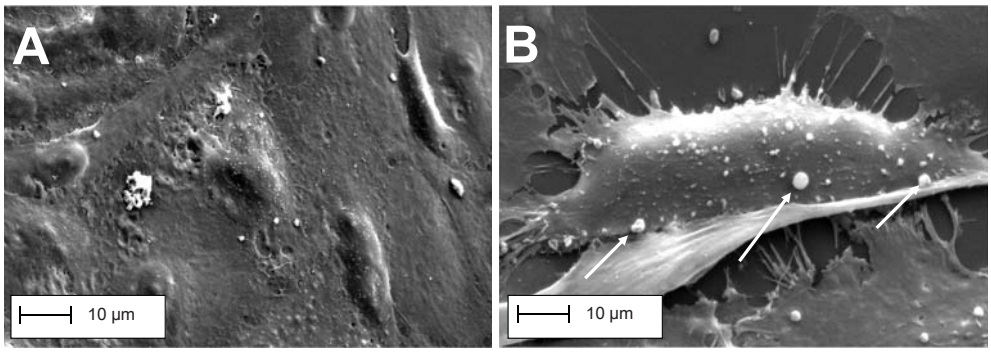

Figure 1b.3 Scanning electron microscope images showing unstimulated cultured human umbilical vein endothelial cells (A) and the formation of microparticles after stimulation of the cells with interleukin-1α (B).

Mechanisms of microparticle formation: activation and apoptosis

It is generally accepted that all eucaryotic cells release microparticles. Microparticle formation *in vitro* occurs whenever a stimulus is applied that induces either cell activation or apoptosis. To date, however, it is unclear whether the mechanisms underlying microparticle formation are identical during these two conditions are identical.

Cell activation

Platelets can be activated by different agonists that bind to specific receptors on the platelet membrane. Thus, stimuli such as thrombin, collagen and adenosine diphosphate (ADP) activate specific transmembrane receptors that transmit signals into the cell. These signals induce changes in second messenger concentrations that in turn modulate cellular responses.[6,7] Stimulation of platelets by these agents not only leads to platelet aggregation and secretion, but also results in membrane vesiculation and the release of microparticles. Alternatively, agents, such as calcium ionophores, trigger microparticle release by directly changing the intracellular concentrations of second messenger molecules. PMP are also formed during prolonged storage of platelets, or when platelets are exposed to high shear-stress conditions *in vitro*.[5] The latter conditions resemble those occurring *in vivo* at stenoses of the vascular tree.

Although the molecular mechanisms underlying microparticle formation are as yet unresolved, the increase in intracellular levels of calcium ions, resulting in the activation of enzymes such as calpain, play an important role.[6,7] Calpain degrades cytoskeletal proteins, and its inhibition partly prevents collagen- and thrombin-induced microparticle formation.[30]

Apoptosis

Programmed cell-death or apoptosis is associated with the abolition of the phospholipid asymmetry of the plasma membrane and condensation of the nucleus, followed by DNA fragmentation and the release of apoptotic blebs or microparticles.[8] The intracellular enzyme family of caspases plays an important role in apoptosis.[8] The irreversible step in which procaspase 3 (CPP32) is converted into the active caspase-3 is regarded as fundamental in the apoptotic process. Caspase-3 activates rho-associated kinase (ROCK I) resulting in the release of apoptotic membrane vesicles, which can also contain DNA fragments.[31]

Functional characteristics of microparticles

The most frequently described characteristic of both *in vitro* and *in vivo* microparticles is their procoagulant activity. Recent observations, however, also suggest their involvement in inflammatory processes,[27] in the transfer of bioactive molecules to other cells and microparticles[9] and the inhibition of endothelium-dependent vasodilatation.[32,33] Not all the properties of cellular microparticles should necessarily be regarded as noxious: specific microparticle subpopulations may even prevent vascular damage. Thus, *in vitro* generated PMP were shown to enhance the activation of protein C, thus facilitating the inhibition of coagulation factors Va and VIIa and preventing thrombin formation.[34] Other microparticle fractions were reported to induce cellular growth, chemotaxis, apoptosis and the outgrowth of transplanted haematopoetic stem cells.[35,36]

The various functional characteristics of *in vitro* generated microparticles as well as of those isolated from the circulation of various patient populations will be discussed (see also Table 1b.1).

Microparticles and coagulation

Coagulation activation plays an essential role in the development of atherothrombosis. Subjects with a high risk of cardiovascular disease show various degrees of hypercoagulability. Coagulation activation requires plasmatic coagulation factors, calcium ions and a procoagulant membrane surface. An essential characteristic of such a suitable surface is the exposure of negatively charged phospholipids. As previously stated, the exposure of such phospholipids is one of the characteristics of microparticles. Coagulation factors bind – via their negative Gla-domains – to the negatively charged phospholipids in the presence of calcium ions, thus forming tenase- and prothrombinase-complexes. PMP expose more binding sites for factors Va, VIIIa, and IXa per unit of membrane surface area than activated platelets. Thus, at least *in vitro*, thrombin formation is supported more efficiently by microparticle membranes than by platelet membranes when corrected for unit surface area.

Table 1b.1 Characteristics of *in vitro* and *in vivo* generated microparticles

Characteristics
***In vitro* generated platelet microparticles:** • stimulate CD11b expression on leukocytes, leukocyte-leukocyte interactions, phagocytosis • induce CD11a/CD18 and CD11b/CD18 on monocytes, resulting in monocyte adhesion to endothelial cells • induce ICAM-1 exposure on endothelial cells, resulting in monocyte adhesion to endothelial cells • stimulate COX2-expression in monocytes and endothelial cells • stimulate thrombocyte aggregation, intracellulair calcium flux, inositol phosphate formation • stimulate protein kinase C, mitogen-activated protein (MAP) kinases and stress (JNK) kinases • transcellular transfer of arachidonic acid, resulting in amplification and modulation of platelet activation • transfer of various cytokine- and chemokine-receptors to haematopoetic and malignant cells • transfer of CXCR4-receptors for HIV-1 virus to cells • enhance engraftment of transplanted bone marrow cells • enhance APC-catalyzed inactivation of Factor Va • co-localise plasminogen-activator inhibitor-1 and vitronectin
***In vivo* circulating platelet microparticles:** • initiate and propagate coagulation / enhance thrombin formation • expose P-selectin • expose tissue factor • transfer tissue factor to other cells and cell-derived microparticles
***In vitro* generated endothelial cell microparticles:** • induce monocyte adhesion to endothelial cells • activate neutrophils • initiate and propagate coagulation / enhance thrombin formation • expose matrix metalloproteinases-2 and -9, induce matrix degradation and angiogenesis
***In vivo* circulating endothelial cells microparticles:** • inhibit endothelium-dependent vasodilation • initiate and propagate coagulation / enhance thrombin formation • are associated with type 1 diabetic microalbuminuria
***In vitro* generated leukocyte microparticles:** • expose tissue factor, transfer tissue factor to platelets and their microparticles • activate endothelial cells and stimulate the secretion of IL-6 via stress-associated signal routes (JNK1)
***In vivo* circulating leukocyte microparticles:** • are present in human atherosclerotic plaques, in close association with tissue factor • are associated with type 2 diabetic microvascular damage

The procoagulant activity of microparticles can be quantified using the thrombin generation test.[13,14] In this assay, the conversion over time of a specific chromogenic substrate by thrombin is measured photospectrometrically. In this system, microparticles supply the procoagulant surface and a possible initiator of coagulation, e.g. tissue factor, and plasma provides the necessary coagulation factors. By adding calcium ions, (activated) coagulation factors can bind to the (microparticle) membranes to initiate and / or facilitate coagulation. In this assay, the generation of thrombin is completely dependent on the presence of microparticles, and in their absence no coagulation occurs.

In vivo, coagulation is initiated by tissue factor, a transmembrane protein that binds factor VII(a) and catalyses its autoactivation. In turn, the tissue factor/factor VIIa complex directly activates factor X to factor Xa. Factor Xa, in the presence of its cofactor Va, forms the prothrombinase complex that converts factor II (prothrombin) into IIa (thrombin). Alternatively, the tissue factor/factor VIIa complex activates factor IX into factor IXa. Together with its cofactor, factor VIIIa, factor IXa forms the tenase complex that subsequently activates factor X into factor Xa. In this system, there is an important role for coagulation factor XI. Minute quantities of thrombin can activate factor XI into factor XIa. Subsequently, factor XIa activates factor IX into factor IXa, thereby enhancing the formation of thrombin.

In vitro, microparticles can both initiate and propagate coagulation.[21,37,38] However, the mechanisms by which *in vivo* microparticles support coagulation *ex vivo* were highly dependent on the clinical conditions. For instance, thrombin formation by microparticles from blood of a patient with meningococcal sepsis and diffuse intravascular coagulation (DIC) was completely inhibited by antibodies directed against either tissue factor or factor VII.[17] These antibodies also completely inhibited thrombin generation by microparticles from human pericardial blood, i.e. blood that collects in the pericardial cavity during coronary artery bypass grafting (CABG).[16] In contrast, neither of these antibodies inhibited thrombin generation initiated by microparticles obtained from healthy subjects. Thrombin generation by these microparticles as well as thrombin generation by microparticles from patients with sepsis and multiple organ failure was mediated by factor XI and in some patients also by factor XII.[13,18] Only recently it was discovered that also tissue factor-independent mechanisms are able to initiate coagulation. One example is the binding of factor X to the monocytic protein Mac-1 (CD11b/CD18) and the subsequent activation of factor X into factor Xa by catepsin G.[39] Possibly, microparticles may also use similar tissue factor-independent, mechanisms to initiate coagulation.

An important question is whether microparticles are procoagulant *in vivo*. This issue is not easily resolved, but several lines of evidence suggest that microparticle-mediated coagulation is indeed clinically relevant. Firstly, microparticles from various patient populations support coagulation *in vitro*.[13,16-18] Secondly, the presence of highly procoagulant, tissue-factor exposing microparticles in certain disease conditions coincided with strongly elevated levels of *in vivo* coagulation activation markers, such as prothrombin fragment F_{1+2} and thrombin-antithrombin complexes. Examples are microparticles from a patient with fullminant DIC and meningococcal septic shock, microparticles from pericardial cavity blood during CABG, and microparticles from synovial fluid from patients with rheumatoid arthritis.[16,17,27] Thirdly, numerous studies

demonstrated an association between elevated numbers of microparticles and the increased risk of thromboembolic complications.[10,19,21,40] Fourthly, an increased bleeding tendency and decreased levels of circulating microparticles have been described in several rare syndromes.[41-43] Finally, direct infusion of artificial phospholipid vesicles in baboons caused severe DIC,[44] and systemic administration of microparticles in rats resulted in thrombus formation.[45]

Microparticles and inflammation

Like coagulation, inflammatory processes underlie the pathogenesis of atherothrombotic vascular disease.[46] Elevated plasma levels of acute phase reactants and other markers of inflammation occur in various high-risk patient populations.[14,38] Microparticles can directly activate and stimulate cells to produce inflammatory substances mediators such as cytokines.[47-49] In addition, at least *in vitro* microparticles mediate intercellular interactions.[49-51] Finally, subpopulations of microparticles isolated from human plasma expose C1q, C3 and C4, strongly suggesting their direct involvement in activation of the complement system.[52] Currently, the relation between cellular microparticles and C-reactive protein (CRP) is studied. This acute phase protein is known to bind to membranes and, in the membrane-bound form, may activate the classical pathway of the complement system, ultimately leading to vascular damage.

Tabel 1.1 lists the reported cell-microparticle and microparticle-microparticle interactions. At present, however, there is no direct evidence that microparticles are involved in inflammatory disease *in vivo*. Although elevated levels of microparticle subpopulations are present in the circulation of patients with inflammatory disease, both of infectious and autoimmune origin, a causal relationship between microparticles and inflammatory processes cannot readily be established, because cytokines trigger cells, thereby stimulating the release of microparticles, whereas microparticles trigger cells to produce and release cytokines.[47] Therefore, it is as yet unclear whether cellular microparticles are a cause or consequence of inflammatory processes and the associated vascular damage.

Microparticles and endothelial cell functions

In vitro microparticles adhere to endothelial cells and subsequently stimulate these cells to produce cell-specific adhesion cell molecules, cytokines and tissue factor.[9] Also, *in vivo* microparticles were found to influence endothelial functions *ex vivo*: microparticles from patients with acute coronary syndromes directly impaired endothelium-dependent vasodilatation in rat aorta-rings, presumably by inhibition of the nitric oxide (NO)-

mediated signal transduction.[32] Also microparticles from women with preeclampsia impaired the endothelium-dependent vasodilatation.[33] Several studies also suggest a relationship between circulating microparticles and endothelial function. Patients with complicated diabetes mellitus, who were treated with a platelet aggregation inhibitor, lowered the numbers of circulating PMP and decreased plasma concentrations of vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1).[38] Conversely, stimulation of endothelial cells *in vitro* by TNFα induced the formation of microparticles exposing adhesion cell molecules, including ICAM-1, E-selectin, vitronectin-3 and platelet-endothelial cell adhesion molecule-1 (PECAM-1). In patients with various systemic and autoimmune diseases elevated levels of microparticles originating from endothelial cells were found.[53-55]

Microparticles and signal transduction, growth, angiogenesis and metastasis

Microparticles may expose adhesion cell molecules, specifically adhere to e.g. endothelial cells and stimulate these cells to produce various intermediates, such as E-selectin and tissue factor.[35,49,56] The actual "communication" between microparticles and cells may occur through transfer of bioactive molecules such as arachidonic acid. Thus, PMP treated with secretory phospholipase A2, an acute phase reactant, had elevated (intravesicular) concentrations of arachidonic acid and these PMP were shown to activate endothelial cells by the transfer of this arachidonic acid.[9,48]

In patients with type 2 diabetes we previously described elevated numbers of microparticles from platelets, granulocytes and lymphocytes that exposed tissue factor.[14] Both *in vitro* and *in vivo* studies demonstrated the presence of tissue factor-positive PMP subpopulations that concurrently expose antigens originating from granulocytes, monocytes or lymphocytes, suggesting a possible transfer of tissue factor by, as well as to these PMP.

PMP have the ability to transfer the CXCR4 receptor from CXCR4-positive to CXCR4-negative cells.[57] This receptor is mandatory for the HIV-1 virus to enter cells, suggesting a role for PMP in the dissemination of HIV-1 particles. Also, microparticles transfer cytokine- and chemokine receptors to haematopoetic, but also to malignant cells, by which mechanism these vesicles may modulate cellular activation, proliferation, survival, apoptosis and chemotaxis.[58] Adherence of PMP to transplanted bone marrow cells stimulated their outgrowth, which may be regarded as a beneficial effect of the PMP. Conversely, in addition to activated platelets, PMP are involved in paraneoplastic thromboembolic complications and metastasis. Only recently, matrix metalloproteinases -2 and -9, enzymes that play a role in matrix degradation and angiogenesis, were detected in microparticles of endothelial origin.[59]

Taken together, cellular microparticles may be carriers of antigens and receptors, including tissue factor, E-selectin and VCAM-1, all of which were previously regarded as "soluble" in plasma. Plasma concentrations of these substances are widely used as measures of endothelial dysfunction in human. By assessing the co-localization of these proteins with cell-specific antigens on microparticles and by measuring the plasma levels of these substances before and after centrifugation (i.e. after removal of the microparticle fraction), it becomes possible to determine the real cellular origin of these antigens, which currently are all ascribed to endothelial cells.

Clinical relevance of cellular microparticles

The last 5 years a growing number of publications appeared reporting elevated numbers of microparticle subpopulations in association with various disease states as well as studies investigating the composition and functional characteristics of microparticles. To date, however, it is unclear whether microparticles are a cause or merely a consequence of metabolic and vascular disease.

Platelet-derived microparticles (PMP)

The clinical relevance of PMP may be illustrated by the rare hereditary Scott syndrome, a disease characterized by a bleeding tendency and a decreased formation of PMP.[41,60] The diminished formation of PMP is caused by a signal transduction defect that diminishes the transmembrane migration and exposure of PS. Castaman's disease and Glanzmann's thrombastenia are other rare syndromes, in which an increased bleeding tendency is associated with a decreased release of PMP.[42,43] Low numbers of circulating microparticles were found in patients with sepsis and these showed an inverse correlation with markers of *in vivo* coagulation.[18] Conversely, elevated numbers of PMP were found in patients suffering from diseases associated with an increased risk of thromboembolic processes and vascular damage, including ITP,[10] acute coronary syndromes,[15,19] acute cerebrovascular disease,[40] heparin-induced thrombopenia (HIT),[37] peripheral arterial disease,[61] complicated diabetes mellitus,[38] severe hypertension,[20] end-stage renal disease,[21] multiple sclerosis,[54] and malignancy.[58] In some studies, also the procoagulant activity of the *in vivo* microparticles, predominantly PMP, was demonstrated. In patients with uncomplicated type 2 diabetes mellitus, we found elevated numbers of tissue-factor-exposing PMP.[14] Unexpectedly, this microparticle-associated tissue factor did not show procoagulant activity and therefore it was hypothesized that this tissue factor may play a role in other processes, such as angiogenesis, growth and signal transduction.

Endothelial-cell microparticles

Increased numbers of microparticles from endothelial cells were reported in patients with acute coronary syndromes, but also in those with severe hypertension, thrombotic thrombocytopenic purpura, systemic lupus erythematosus (SLE) and multiple sclerosis (Table 1b.2). Decreased numbers of endothelial-cell microparticles were measured in subjects with sepsis and multi-organ failure. Some authors explain their occurrence by apoptosis whereas others regard these vesicles as a result of endothelial cell activation. In a recent study, increased levels of endothelial cell microparticles were associated with albuminuria in subjects with type 1 diabetes mellitus, but not in those with type 2 diabetes.[22] We found similar numbers of endothelial cell-derived microparticles in patients with uncomplicated type 2 diabetes and in healthy controls.[14] To note, since in the studies published different endothelial cell markers were used in various study populations,[13,14,19,22,26,53,55] the findings cannot be readily compared.

Table 1b.2 Circulating non-platelet microparticles in diseases with vascular involvement

Cellular origin	Disorder	Microparticle numbers
granulocytes	sepsis / multi organ failure	↑
	type 2 diabetes mellitus*	↑
	preeclampsia	↑
monocytes	atherosclerotic plaques	↑
	type 2 diabetes mellitus	↑
	lung cancer	↑
endothelial cells	systemic lupus erythematosus	↑
	acute coronary syndromes	↑
	congestive heart failure	↑
	sepsis	↓
	thrombotic thrombocytopenic purpura	↑
	multiple sclerosis	↑
	type 1 diabetes mellitus	↑
	severe hypertension	↑
lymphocytes	HIV	↑
	atherosclerotic plaques	↑
	type 2 diabetes mellitus*	↑
	preeclampsia	↑

*The proportion of granulocyte- and lymphocyte-derived microparticles that exposed tissue factor.

Granulocyte-, monocyte- and lymphocyte microparticles

There are but a few studies investigating the numbers of granulocyte-, monocyte- and lymphocyte microparticles in relation to human disease (Table 1b.2). A strong increase in granulocyte-derived microparticles was found in patients with meningococcal sepsis at admission, but after 2 days their number dropped and a concurrent increase in monocytic microparticles was observed.[17] The subject with the most fullminant course of disease developed DIC, which was associated with an extraordinary high number of circulating microparticles of monocytic origin that exposed highly procoagulant tissue factor. High numbers of granulocyte microparticles were also detected in patients with multi organ failure. In patients with type 2 diabetes, monocyte-derived microparticles were associated with plasma E-selectin levels and the highest microparticle numbers were found in subjects with diabetic nephropathy.[62] Women with preeclampsia had elevated numbers of microparticles from granulocytes and lymphocytes (CD4+, CD8+) compared to controls, and – as mentioned before – the microparticles from these patients impaired endothelium-dependent vasodilatation *in vitro*.[33] In HIV-infected patients, elevated numbers of lymphocyte (CD4+) microparticles were measured, suggesting increased apoptosis of CD4-lymphocytes in these patients.[28] An interesting finding is the presence of tissue factor in the vicinity of monocyte- and lymphocyte microparticles in human atherosclerotic plaques, indicating the importance of microparticles in the development of atherosclerosis.[23]

SUMMARY AND DISCUSSION

Microparticles from various cell types – but predominantly from thrombocytes – occur in the human circulation. Elevated numbers of circulating microparticles are found in patients who suffer from diseases associated with an increased thromboembolic risk and vascular damage. Microparticles initiate and propagate coagulation by exposing negatively charged phospholipids on their membrane surface. In addition, under certain conditions, microparticles also expose tissue factor, the initiator of coagulation. The clinical relevance of the presence of microparticles in the circulation of healthy subjects is as yet unclear, but it may be regarded as a reflection of the dynamics between resting, activated and apoptotic cells. In addition, the numbers of circulating microparticles also reflect the result of their production and clearance. In vascular disease states it still remains to be elucidated whether microparticles are a cause or a consequence of the condition, since disease-related factors such as infectious agents, cytokines and metabolic disturbances are known to trigger microparticle formation. Still, it may be assumed that

microparticles do contribute to the severity of disease, as they can disseminate procoagulant and proinflammatory activities throughout the body. Therefore, microparticles may be viewed as part of a cascade of reactions in response to a stimulus. This stimulus that led to their generation determines their numbers, size, biochemical composition and functional characteristics.

Although microparticle formation may be regarded as an adaptive process, such as e.g. the classical inflammatory response, an overshoot of this response, i.e. an excessive release of microparticles, may become harmful to the organism and as such unwanted. Conversely, defective microparticle formation, in particular of PMP, may result in an increased bleeding tendency. Patients with a haemorrhagic trait due to congenital or acquired forms of platelet abnormalities can be treated with plasma cryoprecipitate. The therapeutic efficacy of cryoprecipitates is in part attributed to their content of high concentrations of PMP.[63]

Various anti-platelet drugs, including the GPIIb/IIIa receptor antagonist abciximab[64] and the cAMP phosphodiesterase inhibitor cilostazol,[38] offer therapeutic possibilities, as they reduce excessive PMP formation. Short-term administration of vitamin C at a high dose reduced the number of circulating endothelial-cell-derived microparticles in patients with congestive heart failure.[65] The possible beneficial effect of anti-oxidants was recently also demonstrated by an anecdotal observation, in which consumption of a flavinoid-rich cocoa beverage reduced circulating numbers of PMP in healthy subjects.[66]

Future research should provide insight into the factors that induce microparticle formation and the molecular mechanisms underlying the process of generation of these vesicles, i.e. activation and apoptosis. Collectively, the data obtained from these studies should provide answers to the question as to whether cellular microparticles play a causative role in the development of thromboembolic complications and vascular damage in humans.

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1c

Aims and outline of this thesis

The topic of this thesis focuses on the role of the postprandial state and cell-derived microparticles, and their interaction, in the development of cardiovascular disease (CVD), in particular in high-risk patients.

As previously reviewed in **Chapter 1a**, clinical and epidemiological evidence indicates that postprandial plasma glucose- and triglyceride responses are associated with increased CVD risk. In particular, postprandial triglyceride-rich lipoproteins seem to lead to oxidative stress, endothelial dysfunction, coagulation activation and increased inflammation, all processes involved in atherogenesis. However, all of previously described postprandial studies investigated the effect of a single meal (or rather a single oral fat- or glucose load), with the subject kept fasted for the subsequent 8 h. Also, in some studies, the meals were rather artificial, including (ice-) cream, fluids and high-bulk carbohydrates, whereas in real life, humans consume on average 3 consecutive mixed meals a day.

In view of their functional properties, microparticles may be an important intermediate in the cascade of cellular and plasmatic dysfunctions underlying the process of atherosclerosis, as described in **Chapter 1b**.

The effect of prolonged (i.e. circadian) postprandial metabolic derangements on the physiochemical properties of lipids, on microparticles and concomitant changes in endothelial and vascular functions in subjects with the metabolic syndrome and patients with type 2 diabetes is unknown. We hypothesized that monitoring metabolic changes during a prolonged time period in healthy controls, subjects with the metabolic syndrome and patients with type 2 diabetes, given at least 2 consecutive high-fat mixed meals, thereby mimicking the real-life situation, will unmask abnormalities which are underestimated during fasting conditions or following a single meal. Furthermore, we postulated that these metabolic abnormalities may induce the formation of (specific subpopulations of) microparticle formation, may stimulate the production of inflammatory mediators and oxidative stress, as well as result in coagulation activation and impair endothelial function. By studying both subjects with the metabolic syndrome versus patients with type 2 diabetes, this may reveal the impact of hyperglycemia on top of the other components of the metabolic syndrome.

Since the liver is crucial in lipid metabolism and hepatic steatosis is acknowledged as a key feature of the metabolic syndrome, the amount of liver fat content may contribute to postprandial dyslipidemia. In **Chapter 2**, we studied the effect of 3 consecutive high fat meals on lipid and apolipoprotein responses and its association with liver fat content in males with type 2 diabetes and those with and without the metabolic syndrome.

In the postprandial state, not only derangements in lipid concentrations may occur, but also the physiochemical properties, i.e. composition, size and function of HDL and LDL particles may be affected, that could further promote atherogenesis. In **Chapter 3** we describe the effects of 3 consecutive meals on the physiochemical properties of HDL and LDL particles in type 2 diabetic males and men with and without the metabolic syndrome. In addition, the contribution of liver fat accumulation to postprandial triglyceride-enrichment of HDL particles, and subsequent HDL dysfunction and endothelial dysfunction were studied in **Chapter 4**.

The effect of consecutive meals and the associated metabolic derangements on the formation of microparticles and their possible effects on endothelial function and coagulation activation is unknown. Therefore, in **Chapter 5** we first studied the effects of 2 consecutive high-fat mixed-meals, consumed at an interval comparable to the real-life situation, on postprandial metabolic changes and their association with microparticle generation and endothelial function in healthy males. In addition, to which extent quantitative and qualitative characteristics of microparticles are associated with coagulation in the postprandial state is described in **Chapter 6**.

Second, in **Chapter 7** we describe the effects of exposing males with and without the metabolic syndrome to 3 consecutive high-fat mixed meals, given during a 24-h period, to disclose the full scope of their compromised metabolism and subsequent circulating microparticles and arterial stiffness. Furthermore, in **Chapter 8**, using the same study design, we assessed the effect of postprandial metabolic changes on the numbers and cellular origin of microparticles, and their association with endothelial dysfunction in patients with uncomplicated type 2 diabetes and healthy controls.

In addition, in **Chapter 9**, we review not only the potentially harmful role microparticles may play in the development of CVD, but also present recent evidence regarding their involvement in the maintenance and preservation of cellular homeostasis.

Finally, in **Chapter 10** the results of the abovementioned studies and future directions are discussed.

2

Postprandial lipid and apolipoprotein responses following three consecutive meals associate with liver fat content in type 2 diabetes and the metabolic syndrome

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ABSTRACT

Objective: Liver fat is associated with dyslipidemia following a fat load. Previous studies demonstrated that alimentary fat is temporarily retained within enterocytes and mobilized by subsequently ingested nutrients. As this potentially contributes to cumulative postprandial hyperlipidemia, we assessed postprandial lipoprotein changes and their association with liver fat following 3 consecutive meals during a 24h period in males with type 2 diabetes, and men with the metabolic syndrome (MetS).

Methods: Plasma lipids were measured in 14 type 2 diabetic, 14 MetS and 14 healthy age-matched males, following a standardized breakfast (t=0h), lunch (t=4h) and diner (t=8h). Blood samples were collected before and at t=2, 4, 6, 8, 12, 16, 20 and 24h following breakfast. Liver fat was measured by proton magnetic resonance-spectroscopy.

Results: Type 2 diabetic (mean age 55(4.2)yrs; HbA1c 7.2(1.1)%) and MetS men had similar BMI, waist, blood pressure and triglycerides. 24h-AUC triglycerides, ApoB, and cholesterol-rich-remnants, but not ApoB-48, differed significantly among groups (calculated by ANOVA, all $P<0.05$). Liver fat was independently associated with 24h-AUC triglycerides, ApoB and cholesterol-rich-remnants ($r=0.57$, $P<0.001$, $r=0.38$, $P=0.017$; $r=0.48$, $P=0.002$, respectively), but not with 24h-AUC ApoB-48 ($r=0.22$, $P=0.18$).

Conclusions: In type 2 diabetes and the MetS exposure to 3 consecutive meals produced exaggerated 24h triglyceride, ApoB and cholesterol-rich-remnant concentrations, which were closely associated with liver fat. Instead, ApoB-48 peak was delayed in type 2 diabetes, but not related to liver fat. In addition to liver fat, other mechanisms, including local intestinal processes, determine atherogenic postprandial lipoprotein changes following 3 consecutive meals during 24h.

INTRODUCTION

Exaggerated postprandial elevation of plasma triglyceride-rich lipoproteins and the accumulation of cholesterol-rich-remnant particles in type 2 diabetes mellitus and insulin resistant states have been associated with increased atherogenesis and cardiovascular disease (CVD) risk.[1-3] Both intestinal-derived apolipoprotein (apo) B-48 and liver-derived apoB-100 containing chylomicrons contribute to the postprandial triglyceride-rich lipoprotein responses.

Recently it was suggested that intestinal chylomicron production and release is actively controlled in the intestine and affected by insulin resistance.[4,5] The production rate of apoB-48 correlates with insulin levels[6] and is actually increased in type 2 diabetes.[7] Furthermore, in healthy subjects, the intestinal storage of dietary lipids originating from fat contained in the previous meal can be released by a subsequent glucose load, resulting in a peak of atherogenic lipid particles in the plasma.[8] Whether this mechanism influences plasma apoB-48 concentrations following consecutive meals in insulin resistant states like type 2 diabetes or the metabolic syndrome (MetS) is unclear.

The liver plays an important role in (postprandial) lipid metabolism via the production of triglyceride-rich apoB-100-containing lipoproteins, and their (competitive) clearance by remnant receptors. Non-Alcoholic Fatty Liver Disease (NAFLD) is associated with hepatic insulin resistance, fasting hypertriglyceridemia, unsuppressed postprandial apoB-100 production and VLDL output, and low HDL-cholesterol. In addition, NAFLD is closely related to the MetS and type 2 diabetes.[9,10] Also, it has been demonstrated that postprandial triglyceride-rich lipoprotein concentrations, including apoB-48, following a single meal are associated with NAFLD.[11]

The sequence of daylong meals produce sustained triglyceride increase in type 2 diabetes.[12] Our hypothesis is that the above mechanisms (increased assembly and production, postponed release or retainment, and/or impaired clearance of intestinal-derived triglyceride-rich lipoproteins) will delay apoB-48 peak after consecutive meals in insulin resistant subjects and patients with type 2 diabetes. As a consequence, the use of one single fat load as performed in most studies addressing mechanisms contributing to postprandial lipid responses in type 2 diabetes, will not be sufficient to fully unveil the 24-hour course of the various lipid fractions, and therefore not the full spectrum and/or potential of the atherogenicity of postprandial dyslipidemia.

Using proton magnetic resonance spectroscopy (1H-MRS) and 3 consecutive high-fat mixed meals during 24 hours, we quantified liver fat content[13] and the plasma triglyceride-rich lipoproteins apoB-100 measured as total apoB, apoB-48 and cholesterol-remnants

responses, respectively, and assessed their associations in males with type 2 diabetes, males with the MetS and healthy controls.

MATERIALS AND METHODS

Subjects

Forty-two Caucasian males, aged 40-65 years, with type 2 diabetes (n=14) and with the MetS (n=14), and 14 age-matched healthy males were recruited by advertisement and studied after obtaining written informed consent. Diet, sulphonylurea and/or metformin were the only glucose-lowering treatments allowed in the type 2 diabetic group. Males with the MetS had to meet 3 out of 5 inclusion criteria based on NCEP/ATP III-criteria,[14] without having hyperglycemia during a 75g oral glucose tolerance test (OGTT). Claustrophobia, excess alcohol intake (>20 units/wk), history of hepatitis and/or pancreatitis, abnormal liver and renal function tests (>2 times upper limits of normal), recent (<3 months) changes in weight (≥5%) and/or medication, history or current use of glucocorticosteroids, insulin and/or thiazolidinediones, were exclusion criteria. Since it has been demonstrated that statins reduce postprandial lipid excursions in several patients groups, including the MetS,[15] we excluded participants using statins or other lipid-lowering drugs. Subjects were instructed to omit their medication during the examination and to refrain from heavy physical activities during the previous 24-h. The local ethics committee approved the study and the investigation conformed to the principles outlined in the Declaration of Helsinki.

Study design

After an overnight fast, subjects were admitted in the research unit for a 24-h period and received 3 consecutive, isocaloric (900 kcal), mixed meals (75g carbohydrates, 50g fat (60% saturated), 35g protein), at time points 9.00 AM (breakfast), 1.00 PM (lunch), and 5.00 PM (diner). Breakfast consisted of an EggMcMuffin®, croissant with butter and marmalade, 200 ml of milk, combined with 20 ml of cream, and 13 ml of syrup. The lunch consisted of a Quarterpounder®, croissant with butter, 200 ml of milk, and 16 ml of syrup. Diner consisted of a Quarterpounder®, 90 gr of French fries, 175 gr of salad and 200 ml of water. The subjects were instructed to consume each meal within 15 minutes. Blood samples were drawn before and 2, 4, 6, 8, 12, 16, 20 and 24 hours after breakfast. To avoid lipoprotein lipase (LPL) activation by physical activity, subjects remained in the semi-recumbent position during the whole testing day.

Biochemical measurements

Plasma glucose concentrations were measured by hexokinase-based technique (Roche diagnostics, Mannheim, Germany) and insulin concentrations by immunoradiometric assay (Centaur, Bayer Diagnostics, Mijdrecht, The Netherlands). Plasma total cholesterol, HDL-cholesterol and triglycerides were determined by enzymatic methods (Modular, Hitachi, Japan). LDL-cholesterol was calculated by the Friedewald formula. Glycated hemoglobin (HbA1c) was measured with cation exchange chromatography (Menarini Diagnostics, Florence, Italy; reference values: 4.3-6.1%). Plasma cholesterol-remnants were determined with immunoseparation assay (Otsuka Pharmaceutical Co, Tokio, Japan). Whereas plasma total apoB-lipoprotein was measured by immunochemical method based on immunoprecipitation at 340 nm (Thermo Electron Ov, Vantaa, Finland), we determined plasma apoB-48 by using a sandwich ELISA method that uses monoclonal antibody raised against apoB-48 C-terminal decapeptide.[16] The homeostasis model assessment of insulin resistance (HOMA-IR), was calculated as described by Matthews et al.[17] as fasting insulin (μU/ml) x glucose (mmol/l)/22.5.

MR-spectroscopy

MR examinations were performed after an overnight fast, using a 1.5-T whole-body system (Sonata; Siemens, Erlangen, Germany) in the supine position with the body-array coil positioned at the upper abdominal region as previously described in detail.[18] In brief, at 3 positions in the liver (right anterior, right posterior and left anterior) a 15 cm^3 volume-of-interest (2.5 x 2.5 x 2.5 cm^3) was positioned, avoiding major blood vessels, intra-hepatic bile ducts and the lateral margin of the liver. User-independent spectral quantification was performed with LCModel (version 6.1).[19] Spectroscopic lipid content is expressed as the percentage of the area under the methyl and methylene peaks relative to that under the water peak. The coefficient of variation between two assessments of liver fat was 4.7%.[18]

Abdominal fat volumes

Visceral adipose tissue area (VAT) and subcutaneous abdominal adipose tissue area (SAT) were measured using MRI as described previously.[18] Quantification of VAT and SAT was performed using an image-analysis program, running on a Sparc10 workstation (Sun Microsystems, Palo Alto, CA). Processing of MRI data and calculations of VAT and SAT were performed by a single experienced investigator, unaware of the metabolic state of the studied subject.

Statistical analysis

Results are presented as the means±SE or medians (interquartile range). Twenty four-hour area under the curve (24h-AUC) were calculated according to the trapezoid rule. Differences between groups were calculated using ANOVA and post-hoc analyses (Bonferroni). Non-normally distributed data were log transformed. The association of liver fat content and postprandial lipidemia was assessed by univariate and multivariate linear regression analyses. A $P<0.05$ was considered statistically significant.

EXPERIMENTAL RESULTS

The diabetic, MetS and healthy groups did not differ significantly with respect to age, total cholesterol, LDL-cholesterol and liver enzymes (calculated by ANOVA, Table 2.1). Diabetic males had higher HbA1c, fasting and 2h-postload plasma glucose concentrations, liver and visceral fat content, and were more insulin resistant than non-diabetic males. MetS and healthy controls were comparable regarding HbA1c and fasting and 2h-postload glucose concentrations, whereas males with the MetS did not significantly differ from the type 2 diabetic males with respect to BMI, waist, blood pressure, HDL-cholesterol and triglyceride concentrations (Table 2.1).

Postprandial plasma glucose concentrations (including 24h-AUC) were similar ($P=1.0$) in both non-diabetic groups, but differed significantly from type 2 diabetic males ($P<0.001$) (Figure 2.1A). Males with the MetS and males with type 2 diabetes had identical 24h AUC insulin responses, that differed significantly from healthy controls ($P<0.001$) (Figure 2.1B).

Plasma triglycerides increased significantly after the meals, reaching the highest concentrations 6 hours after breakfast in all groups (3.4±0.3 mmol/L in diabetic males, 3.4±0.3 mmol/L in males with the MetS and 2.2±0.3 mmol/L in controls, $P<0.001$ in all groups compared to baseline. Plasma triglycerides in the healthy subjects decreased after 6 hours; the third meal did not seem to affect plasma triglyceride levels (2.2±0.3 mmol/L at 6 hours versus 1.5±0.1 mmol/L at 12 hours, $P<0.01$). In contrast, these remained significantly elevated after 6 hours in the MetS and type 2 diabetes group ($P<0.01$, compared to controls). Plasma apoB lipoproteins and cholesterol-rich-remnants concentrations decreased significantly following the third meal in the control group (106.1 (8.3) to 104.3 (7.9) mg/dl, $P=0.027$), but not in the MetS and diabetic males (Figure 2.1).

Table 2.1 Baseline characteristics of the study population

	T2DM	MetS	controls	*P*	T2DM vs MetS, *P*	T2DM *vs* controls, *P*	MetS *vs* controls, *P*
N	14	14	14	-	-	-	-
Age (years)	55.5±1	56.5±2	54.4±2	0.67	1.00	1.00	1.00
BMI (kg/m^2)	32.4±1	30.3±1	26.8±1	<0.001	0.31	<0.001	0.031
Waist (cm)	111.7±3	110.3±3	99.4±2	0.002	1.00	0.003	0.01
SBP (mmHg)	137±3	139±3	122±2	0.001	1.00	0.004	0.001
DBP (mmHg)	83±1	84±2	76±2	0.001	1.00	0.006	0.001
HbA1c (%)	7.1±0.3	5.9±0.1	5.5±0.1	<0.001	0.001	<0.001	0.51
Fasting glucose (mmol/l)	8.6±0.6	5.6±0.1	5.4±0.1	<0.001	<0.001	<0.001	1.00
Post 75 g OGTT (mmol/l)	15.3±3	6.1±0.2	5.1±0.2	<0.001	<0.001	<0.001	0.57
HOMA-IR	3.8±0.5	2.0±0.3	1.0±0.1	<0.001	0.001	<0.001	0.12
Total cholesterol (mmol/l)	5.0±0.1	5.3±0.2	5.1±0.2	0.55	0.84	1.00	1.00
HDL-C (mmol/l)	1.06±0.1	1.09±0.1	1.52±0.1	<0.001	1.00	0.001	0.002
LDL-C (mmol/l)	3.0±0.1	3.3±0.2	3.1±0.2	0.55	0.84	1.00	1.00
Triglycerides (mmol/l)	2.1±0.3	2.1±0.2	1.0±0.1	0.002	1.00	0.006	0.007
apoB (mg/dl)	124±8	128±6	100±8	0.024	1.00	0.091	0.034
apoB-48 (µg/ml)	5.7±1.0	5.1±0.6	4.3±0.6	0.46	1.00	0.66	1.00
RLP-C (mg/dl)	8.7±1.0	8.5±0.5	7.1±0.5	0.23	1.00	0.35	0.49
Liver fat (%)	17.8(7.6-31.7)	7.6(2.9-10.2)	3.9(2.1-7.0)	0.006	0.045	<0.001	0.08
SAT (mm^2)	286±32	303±27	227±20	0.12	1.00	0.37	0.15
VAT (mm^2)	382±24	271±15	242±16	<0.001	<0.001	<0.001	0.84

Values are means±SE or median (interquartile range), *P* value is calculated by ANOVA for 3 groups and by Bonferroni for 2 groups. T2DM, type 2 diabetes; MetS, metabolic syndrome; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; RLP-C, cholesterol-rich-remnants; SAT, subcutaneous abdominal adipose tissue area; VAT, visceral adipose tissue area.

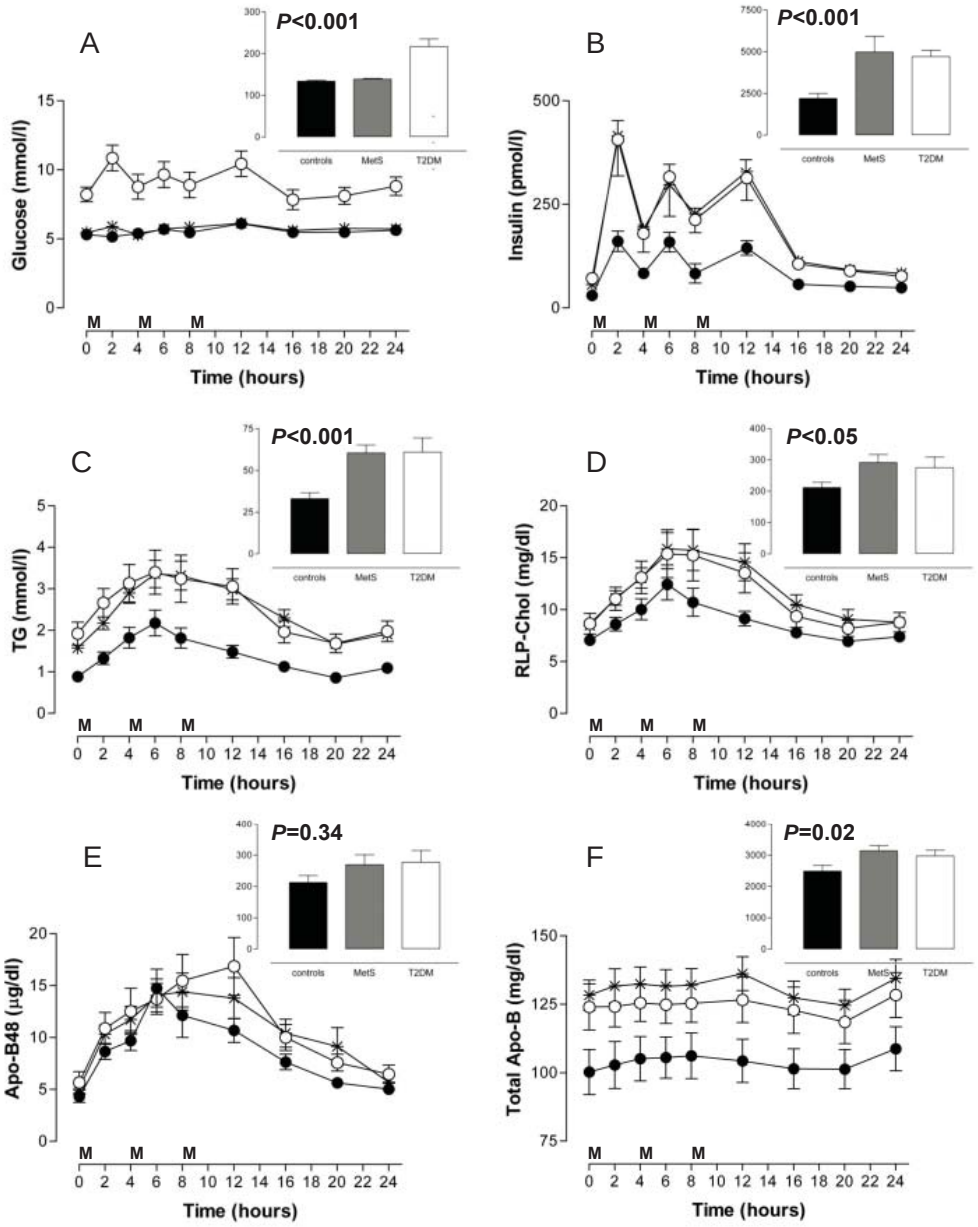

Figure 2.1 The 24-h course of plasma glucose (A), insulin (B), triglycerides (C), cholesterol-rich-remnants (D), apoB-48 lipoprotein (E) and total apoB lipoprotein (F) concentrations after 3 high-fat mixed meals in T2DM (open circles), MetS (asterisk) and healthy males (solid circles). Bars (black, healthy males; grey, metabolic syndrome (MetS); white, type 2 diabetes (T2DM)) in the insets represent respective 24h-AUC values. Meal intake is indicated by M. The *P* value given for 24h-AUC difference (ANOVA). Data are mean±SE.

ApoB-48 concentrations were highest in type 2 diabetic males after the third meal (t=12h), compared to controls (16.8±2.8 versus 10.7±1.1 μg/dl, $P<0.05$).

Twenty four-hour AUCs of apoB lipoproteins and cholesterol-rich-remnants differed significantly (both $P<0.05$), whereas 24h-AUC of apoB-48 lipoproteins did not significantly differ ($P=0.34$, ANOVA) between groups. Interestingly, males with the MetS had the highest postprandial apoB concentrations ($P=0.045$, compared to controls). Twenty four-hour AUCs of apoB and apoB-48 correlated highly with each other ($r=0.71$, $P<0.001$), and with 24h-AUC of cholesterol-rich-remnants ($r=0.67$, $P<0.001$; $r=0.64$, $P<0.001$, respectively).

Liver fat was higher in the diabetic than in the non-diabetic groups (Table 2.1). Posthoc analyses revealed a significant difference in liver fat between diabetic males and MetS males ($P<0.05$). A nearly significant difference was found in liver fat between MetS and controls, $P=0.08$. Liver fat content was positively associated with BMI ($r=0.50$, $P=0.001$), waist ($r=0.49$, $P=0.001$), VAT ($r=0.30$, $P=0.048$), fasting plasma glucose ($r=0.39$, $P=0.001$), 2h-postload glucose ($r=0.65$, $P=0.001$), fasting insulin ($r=0.66$, $P<0.001$), triglycerides ($r=0.55$, $P<0.001$), apoB ($r=0.36$, $P=0.019$), cholesterol-rich-remnants ($r=0.39$, $P=0.011$), and HOMA-IR ($r=0.70$, $P<0.001$). Liver fat content was not associated with SAT, fasting total cholesterol, LDL-cholesterol and apoB-48 concentrations. Additional univariate analyses also showed an association of liver fat content with 24h-AUCs of triglycerides, apoB and cholesterol-rich-remnants ($r=0.57$, $P<0.001$, $r=0.38$, $P=0.017$; $r=0.48$, $P=0.002$, respectively), but not with 24h-AUC apoB-48 ($r=0.22$, $P=0.18$) (Figure 2.2). Since glucometabolic state did not affect the association of liver fat content and postprandial lipoproteins, as excluded by introducing an interaction term in the multivariate analysis, the data of the 3 groups were pooled for the calculation of the regression line (Figure 2.2).

Univariate analyses performed in the 3 separate groups revealed a significant correlation of 24h-AUC of triglycerides with liver fat content in the MetS males only ($r=0.583$, $P=0.047$). Nearly significant associations with liver fat content in healthy controls and type 2 diabetic males were 24h-AUC cholesterol-rich-remnants ($r=0.494$, $P=0.073$ and $r=0.473$, $P=0.087$, respectively), and in type 2 diabetes 24h-AUC of triglycerides (0.476, $P=0.086$). No correlations of apoB-48 with liver fat content in the 3 separate groups could be detected.

Multivariate analysis was performed in the pooled groups to study the independent association of liver fat content with postprandial lipidemia. Twenty four-hour AUC of triglycerides, apoB and cholesterol-rich-remnants were separately entered as a dependent

variable, and subsequently, liver fat content, metabolic state (type 2 diabetes, MetS, or control), age, BMI or VAT, and fasting glucose were entered as independent variables into the model (Table 2.2 for triglycerides). Adjustment for these variables did not change the association of liver fat with 24h-AUC triglycerides. Comparable results were found for apoB and cholesterol-rich-remnants (data not shown). Table 2.3 shows results of forward stepwise multivariate linear regression model. The independent variables in each model were liver fat, metabolic state, age, BMI, waist, VAT, HbA1c, HOMA-IR. Liver fat remained as a significant determinant of 24h-AUC triglycerides, apoB, and cholesterol-rich-remnants, but not of 24h-AUC of apoB-48. In contrast, 24h-AUC of apoB-48 was independently determined by HbA1c.

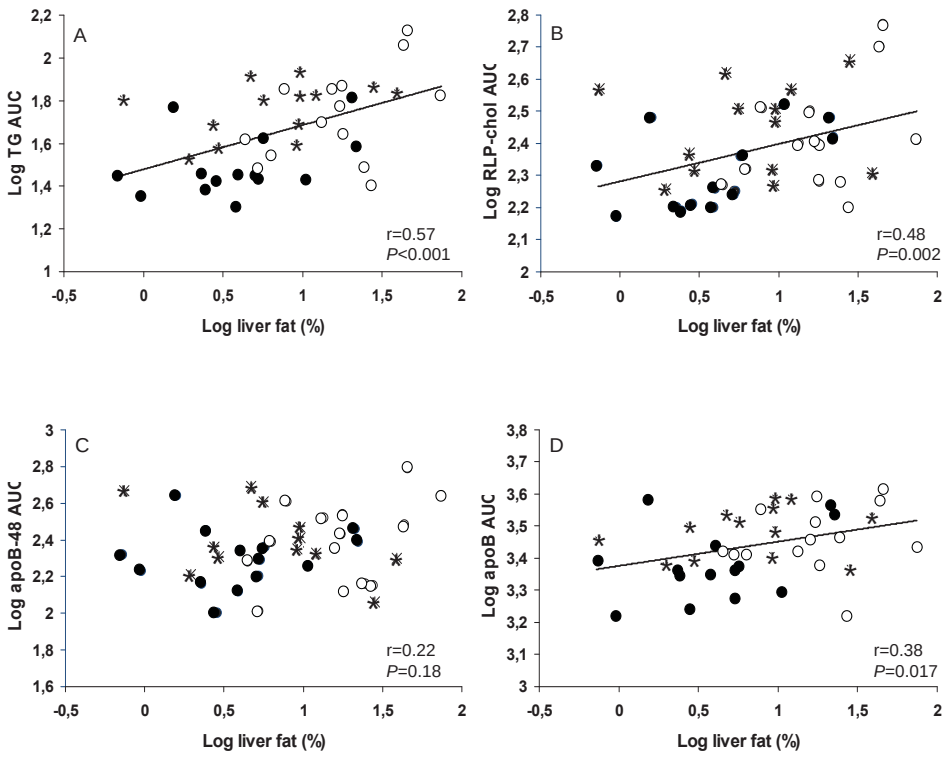

Figure 2.2 Scatter plots representing the relationship between liver fat content and postprandial lipidemia, including triglycerides (A), lipoprotein total apoB (B), lipoprotein apoB-48 (C) and cholesterol-rich-remnants (D) in the whole study population (T2DM (open circles), MetS (asterisk) and healthy males (solid circles)). Pearson correlation coefficients are shown.

Table 2.2 Multivariate associations between liver fat content and 24-h AUC triglyceride

	24h-AUC TG (95% CI)	*P*	*R*	R^2	*P*
Model 1			**0.50**	**0.25**	**0.001**
Liver fat content (log %)	25.910 (13.831 to 43.103)	0.001			
Model 2 (Model 1 + metabolic state)			0.50	0.25	0.001
Liver fat content (log %)	25.910 (8.117 to 42.498)	0.005			
Metabolic state	1.210 (-16.486 to 18.905)	0.891			
Model 3 (Model 2 + age)			0.51	0.26	0.01
Liver fat content (log%)	24.921 (7.558 to 42.283)	0.006			
Metabolic state	1.512 (-16.344 to 19.368)	0.865			
Age	0.406 (-0.785 to 1.598)	0.494			
Model 4 (Model 3 + VAT)			0.53	0.28	0.016
Liver fat content (log%)	24.381 (6.972 to 41.791)	0.007			
Metabolic state	8.412 (-14.231 to 31.054)	0.456			
Age	0.467 (-0.732 to 1.665)	0.435			
VAT	-0.001 (-0.002 to 0.001)	0.321			
Model 5 (Model 3 + BMI)			0.52	0.27	0.023
Liver fat content (log%)	23.403 (4.903 to 41.903)	0.015			
Metabolic state	0.179 (-18.588 to 18.945)	0.985			
Age	0.393 (-0.812 to 1.599)	0.512			
BMI	0.563 (-1.606 to 2.731)	0.602			
Model 6 (Model 4 + HOMA-IR)			0.57	0.33	0.013
Liver fat content (log%)	19.372 (1.143 to 37.600)	0.038			
Metabolic state	2.073 (-21.556 to 25.702)	0.860			
Age	0.464 (-0.711 to 1.639)	0.429			
VAT	-0.001 (-0.002 to 0.000)	0.185			
HOMA-IR	4.915 (-1.362 to 11.193)	0.121			

Table 2.3 Significant correlates of forward stepwise multivariate linear regression model

Dependent variable	Independent correlate	Coefficient	Standard error	Significance
24h-AUC TG	Liver fat	0.883	0.234	0.001
24h-AUC RLP cholesterol	Liver fat	2.650	1.000	0.012
24h-AUC apoB	Liver fat	563.310	220.484	0.015
24h-AUC apoB-48	HbA1c	44.902	17.480	0.014

Variables excluded metabolic state, age, BMI, waist, VAT, (HbA1c), HOMA-IR. TG, triglyceride; BMI, body mass index; HOMA-IR, homeostasis model assessment of insulin resistance; RLP cholesterol, cholesterol-rich remnants; VAT, visceral adipose tissue area.

DISCUSSION

This study demonstrates that the consecutive consumption of 3 fat-rich mixed meals by men with type 2 diabetes and men with the MetS results in exaggerated and prolonged postprandial dyslipidemia during 24 hours, as compared to the profile in healthy age-matched controls. We demonstrate for the first time that postprandial 24h-AUC of atherogenic apoB and cholesterol-rich-remnant particles are independently associated with liver fat content, whereas no such associations were found with 24h-AUC of apoB-48. Another novel finding in the present study is that 3 sequential meals produce significant delay in plasma apoB-48 peak at 8–12 hours in type 2 diabetes as compared to healthy subjects in whom apoB-48 steadily decreased after a third meal.

High levels of apoB-48-containing lipoproteins may contribute to the increase of apoB triglyceride-rich lipoproteins and cholesterol-remnants in the postprandial state, as these triglyceride-rich lipoproteins are cleared by the same pathways. ApoB-48 triglyceride-rich lipoproteins have higher affinity for LPL than apoB-100 triglyceride-rich lipoproteins.[20] Thus when LPL-activity is reduced, as in insulin resistant states,[21] in particular postprandial apoB-100 concentrations may become exaggerated and prolonged due to impaired clearance. Therefore, the high total apoB concentrations we found in the MetS males can be explained by the preferential hepatic clearance of apoB-48 that result in higher apoB-100 concentrations than can be expected on high liver fat content only. Furthermore, an increased production or retainment of apoB-48 chylomicrons by the intestine in type 2 diabetes will result in higher apoB-48 concentrations following consecutive meals. This is in line with the findings by Robertson et al., who showed that apoB-48 lipoproteins were temporarily retained within enterocytes following a fat load and were released after a subsequent glucose load in healthy subjects.[8] Speculatively, the underlying mechanism of this phenomenon will be impaired in type 2 diabetes and result in an delayed release of apoB-48 lipoproteins from the intestine. Other possible intestinal mechanisms that may contribute to our findings are increased microsomal triglyceride transfer protein and diacylglycerol acyltransferase activity, expression of Niemann-Pick C1-like 1, and blunted incretin responses.[22] Current findings agree well with the view that chylomicron metabolism is regulated by multiple steps at the level of the enterocytes.

Our finding that 24h-AUC apoB-48 is not associated with liver fat content seems in contrast to previous findings.[11] However, the latter study separated lipoprotein fractions, but used only one high fat mixed meal, and results were calculated in subjects divided according to liver fat content, irrespective of the diabetic state. Furthermore, a direct comparison between our postprandial responses following breakfast only and the previous results is

not possible, since in our study the second meal was introduced 4 hours after breakfast and the largest postprandial differences occur after 4 hours.

We confirmed previous findings showing a significant difference in liver fat content between normoglycemic males with the MetS compared to those with hyperglycemia (or type 2 diabetes), despite identical BMI, waist and dyslipidemia. This finding is in line with previous observations showing that liver fat accumulation may be different in people with similar BMI and waist circumference.[23] Furthermore, hyperglycemia contributes to liver fat content, by increasing malonyl-CoA that inhibits carnitine palmitoyltransferase-1 leading to a decrease in mitochondrial β-oxidation and shuttling long-chain acyl-CoA into intracellular triglyceride accumulation.[24,25] Our findings are in accordance with the concept that fatty liver secretes high rates of VLDL particles,[26] since liver fat correlated with both fasting and postprandial triglyceride, apoB and cholesterol-rich-remnant concentrations following 3 consecutive meals. Multivariate regression analyses indicate the importance of glycemic control in postprandial apoB-48 metabolism. This finding is in line with recent results by Su et al., who demonstrated an altered postprandial apoB-48 metabolism following 2 sequential meals in normolipidemic subjects with poorly controlled type 1 diabetes.[27]

One of the limitations of the present study was that we did not measure LPL activity or specific apolipoproteins on chylomicrons. Furthermore, we determined total apoB and apoB-48 in plasma and not in lipoprotein fractions. Finally, our study included a relative small number of subjects, which may not allow generalization of the data.

In conclusion, we found that exposure of males with type 2 diabetes and males with the MetS without hyperglycemia to 3 consecutive meals results in exaggerated and prolonged postprandial dyslipidemia during 24 hours, compared to healthy controls. Since the study design closely resembles daily meal patterns, it underscores the significance of postprandial dyslipidemia being a CVD risk factor in the MetS and type 2 diabetes. Furthermore, 1H-MRS-measured liver lipid content was positively and independently associated with postprandial elevations of triglycerides, total apoB and cholesterol-rich-remnants. Consecutive meals reveal a postponed peak response of apoB-48 containing particles in type 2 diabetic males, independently of hepatic fat content.

CONCLUSION

Our findings suggest that in addition to liver fat content, other mechanisms, speculatively intestinal insulin resistance, determine the quantity and quality of postprandial lipoprotein changes following 3 consecutive meals during 24 hours. Future studies should elucidate

possible mechanisms, including intestinal insulin resistance and local impaired intestinal hormone responses, that underlie our observations in order to positively effect postprandial lipidemia as a risk factor for atherogenesis.

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3

Effect of three consecutive meals on the physicochemical properties of HDL and LDL in individuals with the metabolic syndrome and patients with type 2 diabetes

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ABSTRACT

Background/objectives: Postprandial hyperlipidemia, which is exaggerated and prolonged in insulin resistant individuals, has been associated with cardiovascular disease. The objective of the present study was to investigate whether and how the composition, size, and function of HDL and LDL particles are affected in the postprandial state among males with the metabolic syndrome (MetS) or type 2 diabetes (T2DM), compared to controls.

Subjects/methods: Fourteen males with T2DM, 14 with the MetS , and 14 age-matched controls were given 3 standardized high-fat mixed meals (900 kcal; 50g fat, 75g carbohydrate, and 35g protein) as breakfast, lunch and dinner. Blood sampling was performed just before the meals, and 4 and 8 hours after the last meal. HDL and LDL were isolated by ultracentrifugation and analyzed for their composition, particle diameter, and physicochemical properties.

Results: Postprandial triglycerides levels in plasma, HDL and LDL particles increased significantly in all groups ($P<0.01$). Compared to the controls, patients with T2DM had smaller LDL particles, and in agreement, a lower cholesterol-to-protein content in both fasting and postprandial samples. A prolonged increase in susceptibility of LDL to oxidation was found in all subjects, but was most evident in T2DM. The postprandial effect on LDL oxidation was associated with an increase in LDL triglyceride ($r=0.29$, $P<0.05$). In T2DM the anti-oxidative capacity of HDL trended to impairment after the third meal.

Conclusions: Postprandial increases in triglycerides, especially in T2DM, are accompanied by pro-atherosclerotic functional changes in HDL and LDL particles.

INTRODUCTION

Postprandial hyperlipidemia is considered to be an important risk factor for atherosclerosis.[1,2] In clinical practice, however, triglycerides are traditionally measured in the fasting state, typically the lowest triglyceride level of the day. Recent studies have shown that measurement of non-fasting triglycerides levels may be superior to fasting concentrations because the former are clearly associated with increased risk of cardiovascular disease (CVD) and mortality.[3-5] Postprandial responses related to triglyceride metabolism may trigger a number of pro-atherosclerotic processes including leukocyte activation,[6] up-regulation of pro-inflammatory genes,[7] decrease in circulating antioxidant defense,[8] and oxidative stress.[2,9,10] Furthermore, reactive oxygen species (ROS) peroxidize constituents of lipoproteins, leading to modifications, including oxidized LDL and HDL, that may alter their functional properties. Individuals with the metabolic syndrome (MetS) and particularly patients with type 2 diabetes mellitus (T2DM) have postprandial hyperlipidemia which is associated with an increased CVD risk. It is likely that the lipoprotein composition and physicochemical properties may become postprandially more affected in comparison to healthy normolipidemic subjects. The postprandial effects on lipoprotein metabolism have been described in literature, but in contrast to our study most studies used just one meal and no more than one or two study groups. To the best of our knowledge, the present study is the first to examine the influence of 3 consecutive meals on both LDL and HDL properties in men with the MetS or T2DM compared to controls. So, the aim of the present study was to investigate whether postprandial hypertriglyceridemia affects the physicochemical properties of HDL and LDL in individuals with T2DM and with the MetS, in comparison to controls.

MATERIALS AND METHODS

Study design

In total 42 nonsmoking Caucasian male subjects were enrolled; 14 patients with uncomplicated T2DM, 14 subjects with the MetS, and 14 age-matched volunteers (i.e. 'control group'). Diet, sulphonylurea and/or metformin were the only glucose-lowering treatments allowed in the T2DM group. Males with the MetS had to meet 3 out of 5 inclusion criteria based on NCEP/ATP III-criteria,[11] without having hyperglycemia during a 75g oral glucose tolerance test (OGTT). Inclusion criteria for control males included a BMI >25 kg/m^2, normoglycemia during a 75g OGTT, fasting triglycerides <1.7 mmol/l, and blood pressure <145/90 mmHg. Exclusion criteria for all were excess alcohol intake (>20 units/week),

history of hepatitis and/or pancreatitis, abnormal liver and renal function tests (>2 times upper limits of normal), recent (<3 months) changes in weight (≥5%) and/or medication, history or current use of glucocorticosteroids, insulin and/or thiazolidinediones. Since it has been demonstrated that statins reduce postprandial lipid excursions in several patients groups, including MetS,[12] we excluded subjects using statins or other lipid-lowering drugs. Participants were instructed to omit their medication during the examination and to refrain from heavy physical activities during the previous 24-h. After an overnight fast, participants received 3 consecutive, isocaloric (900 kcal) mixed meals (75g carbohydrates, 50g fat [60% saturated], and 35g protein) at time points t=0-h (breakfast), 4-h (lunch), and 8-h (dinner) and were studied during a 16-h period.[13] Venous blood collections were performed before and 4 hours following every single meal (t=0, 4, 8, 12, and 16-h) for measurements of plasma lipids, glucose, insulin and for measurement of the physicochemical properties of HDL and LDL. Plasma aliquots were obtained after centrifugation (1550*g*, 20 min at 4°C), snap frozen in liquid nitrogen, and stored at -80°C until assay. Informed consent was obtained from all participants and the study was approved by the local ethics committee.

Routine biochemical analyses

Glucose was measured enzymatically (Roche, Mannheim, Germany) and insulin with a double-antibody radioimmunoassay (Linco Research, St. Louis, MO, USA). HbA1c was analyzed by ion-exchange high-performance liquid chromatography (Bio-Rad, Veenendaal, The Netherlands). Total-cholesterol, HDL-cholesterol and triglycerides were measured by standard enzymatic methods (Roche, Mannheim, Germany). LDL-cholesterol was calculated, but only in fasting plasma samples.[14] The protein content of LDL and HDL was measured by the Lowry method, modified to allow measurement in lipoproteins as described by Markwell.[15] The concentration of cholesterol and triglycerides in isolated lipoprotein fractions were measured by standard enzymatic methods (Roche, Mannheim, Germany).

HDL and LDL particle size and function

HDL and LDL were isolated by ultracentrifugation and afterwards the average particle size was determined by high performance gel-filtration chromatography, using fibrinogen (22.2 nm), thyroglobulin (17.0 nm) and bovine serum albumin (7.1 nm) as calibrators of known diameter.[16] Intra- and inter-assay CVs were 0.1% and 0.2%, respectively. The susceptibility of LDL to *in vitro* oxidation, expressed as lag time, was determined by monitoring the conjugated dienes formation using copper (II) ions as pro-oxidant at a final concentration of 18 μmol/L.[17] Experiments were performed at 30°C on a HTS 7000 plate reader (Perkin

Elmer, Norwalk, CT, USA). The intra- and inter-assay CVs for lag time determination were 1.6% and 3.6%. The anti-oxidative function of HDL was assessed by monitoring its capacity to inhibit the oxidation of dichlorodihydrofluorescein (Invitrogen, Carlsbad, CA, USA) by oxidized 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (Avanti Lipids, Alabaster, AL, USA).18 Briefly, in a total volume of 790 µL phosphate buffered saline (pH 7.4), 35 µL of a normal LDL solution (final concentration of 18 µmol/L cholesterol), 35 µL of test HDL-cholesterol (final concentration of 11 µmol/L cholesterol), 20 µl oxidized 1-palmitoyl-2-arachidonoyl-sn-phosphatidylcholine (final concentration of 50 µmol/L), and 10 µL of dichlorodihydrofluorescein solution (final concentration of 40 µmol/L) were incubated in glass tubes for 2 hours at 37°C. Afterwards the fluorescence intensity was determined with a HTS 7000 plate reader at 485 and 530 nm (excitation and emission wavelength, respectively). Values for fluorescence intensity at t=0 for each group were normalized to 1.0, and consequently HDL function >1.0 in postprandial samples may indicate dysfunctionality. The intra-assay CV was 3.4%.

Statistical analysis

Data are presented as mean (SD) or median (interquartile range). Non-normally distributed data were log transformed before analysis. Correlations were performed using Spearman's rank correlation test and differences between groups were tested using ANOVA and Bonferroni post hoc analyses. To estimate the overall changes during 16-h, the area under curve (AUC) of metabolic parameters against time was calculated according to the linear trapezoidal method. In order to test whether the changes of the physicochemical properties of HDL and LDL over time are affected by group, i.e. controls, MetS and T2DM, we performed analysis of variance (ANOVA) for repeated measurements with the interaction-term time x group. $P<0.05$ was considered to indicate statistical significance. All analyses were performed using SPSS software, version 15 (SPSS Inc, Chicago, IL, USA).

RESULTS

Baseline characteristics

Subject characteristics at baseline divided by group are illustrated in Table 3.1. No significant differences between the groups were noted in age, fasting total cholesterol and LDL-cholesterol. Individuals with either MetS or T2DM had higher values for waist circumference, blood pressure, insulin and plasma triglycerides. BMI, fasting glucose and HbA1c were significantly higher in the T2DM group. HDL-cholesterol was significantly

Table 3.1 Baseline clinical and biochemical characteristics of the study population

Variable	Controls	MetS	*P* value[a]	T2DM	*P* value[b]
N	14	14		14	
Age, years	54.3 (7.5)	56.4 (6.5)	NS	55.5 (4.0)	NS
BMI, kg/m^2	27.5 (2.9)	30.7 (3.2)	NS	32.7 (4.1)	<0.001
Waist circumference, cm	101.2 (8.4)	111.8 (9.8)	0.012	112.1 (9.2)	0.009
SBP, mmHg	121 (6)	139 (13)	<0.001	136 (13)	<0.001
DBP, mmHg	74 (5)	84 (6)	<0.001	83 (4)	<0.001
Fasting glucose, mmol/L	5.4 (0.2)	5.6 (0.4)	NS	8.8 (2.2)	<0.001
HbA1c, %	5.6 (0.4)	5.9 (0.4)	NS	7.2 (1.1)	<0.001
Insulin, pmol/L	27 (22-37)	51 (36-85)	0.007	66 (41-84)	<0.001
Total cholesterol, mmol/L	5.1 (0.9)	5.4 (0.9)	NS	5.1 (0.5)	NS
HDL-cholesterol, mmol/L	1.49 (0.40)	1.10 (0.31)	0.006	1.06 (0.18)	0.002
LDL-cholesterol, mmol/L	3.1 (0.8)	3.2 (0.8)	NS	3.1 (0.5)	NS
Triglycerides, mmol/L	1.0 (07-1.2)	2.0 (1.7-2.6)	0.002	1.8 (1.3-2.7)	0.002

MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus; BMI, body mass index; DPS, diastolic blood pressure; SBP, systolic blood pressure. Data are presented as mean (SD), or median (interquartile range).
[a]MetS syndrome compared with control group
[b]T2DM compared with control group

lower in MetS and T2DM. Table 3.2 shows the physicochemical properties of HDL and LDL in the three study groups before the meals. The triglyceride content of lipoprotein particles was higher in the T2DM group, but reached statistical significance only in HDL. At baseline, T2DM patients, relative to controls, had smaller LDL particles (P<0.05) and in agreement a lower cholesterol-to-protein content in their LDL (P<0.05). Likewise, the cholesterol content of HDL was significantly lower in patients with MetS and T2DM (both P<0.001), but differences in fasting HDL particle size did not reach statistical significance (P=0.22 and P=0.08, respectively for MetS and T2DM compared to controls).

Effects of meals on plasma lipids, glucose, insulin and lipoprotein properties

The postprandial responses following 3 consecutive meals were shown in Figures 3.1 and 3.2 and were also presented as AUC in Table 3.3. Plasma triglyceride peaked 4 hours (t=4-h) after the first meal in controls, whereas in MetS and T2DM the highest levels were observed just before dinner (t=8-h; Figure 3.1). The increase in plasma triglyceride

Table 3.2 Fasting lipoprotein properties of the study population

Variable	Controls	MetS	*P* value[a]	T2DM	*P* value[b]
N	14	14		14	
HDL constituents and size					
Triglyceride-to-protein, mmol/g	0.07 (0.02)	0.08 (0.03)	NS	0.09 (0.03)	0.018
Cholesterol-to-protein, mmol/g	0.93 (0.15)	0.70 (0.12)	<0.001	0.67 (0.11)	<0.001
Particle size, nm	8.61 (0.35)	8.42 (0.18)	NS	8.38 (0.21)	NS
LDL constituents, size and susceptibility to oxidation					
Triglyceride-to-protein, mmol/g	0.27 (0.07)	0.25 (0.05)	NS	0.30 (0.11)	NS
Cholesterol-to-protein, mmol/g	4.31 (0.33)	4.06 (0.30)	NS	3.93 (0.41)	0.018
Particle size, nm	21.23 (0.55)	20.59 (0.47)	0.016	20.69 (0.62	<0.05
Oxidisabilty, min	63.2 (7.4)	56.8 (7.6)	NS	62.8 (8.7)	NS

MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus. Data are presented as mean (SD).
[a]MetS syndrome compared with control group
[b]T2DM compared with control group

Table 3.3 Postprandial response of 3 consecutive meals on plasma lipids, glucose, insulin, lipoprotein properties, during a 16-h period expressed as area under the curve (AUC)

Variable	Controls	MetS	*P* value[a]	T2DM	*P* value[b]
N	14	14		14	
AUC plasma variables					
Triglycerides, mmol/L	25.3 (11.3)	45.2 (13.6)	0.014	46.1 (24.8)	0.010
HDL-cholesterol, mmol/L	23.1 (6.4)	15.7 (4.1)	<0.001	14.9 (2.6)	<0.001
Glucose, mmol/L	88.2 (6.9)	91.2 (5.0)	NS	145.8 (48.1)	<0.001
Insulin, nmol/L	1.3 (0.7)	3.3 (2.4)	<0.001	2.7 (0.7)	<0.001
AUC HDL constituents and size:					
Triglyceride-to-protein, mmol/g	1.4 (0.3)	1.7 (0.5)	NS	1.8 (0.6)	NS
Cholesterol-to-protein, mmol/g	14.6 (2.8)	10.8 (1.8)	<0.001	10.2 (1.8)	<0.001
Particle size, nm	145 (6)	142 (4)	NS	142 (4)	NS
AUC LDL constituents, size and oxidation					
Triglyceride-to-protein, mmol/g	5.0 (1.1)	4.5 (1.0)	NS	5.3 (1.8)	NS
Cholesterol-to-protein, mmol/g	69.4 (5.1)	64.1 (5.1)	NS	62.4 (7.1)	0.009
Particle size, nm	341 (8)	330 (7)	0.005	332 (10)	0.042
Oxidisabilty, min	987 (105)	898 (112)	NS	951 (133)	NS

AUC, area under the curve over a 16-h time period; MetS, metabolic syndrome; T2DM, type 2 diabetes mellitus. Data are presented as mean (SD).
[a]MetS syndrome compared with control group
[b]T2DM diabetes compared with control group

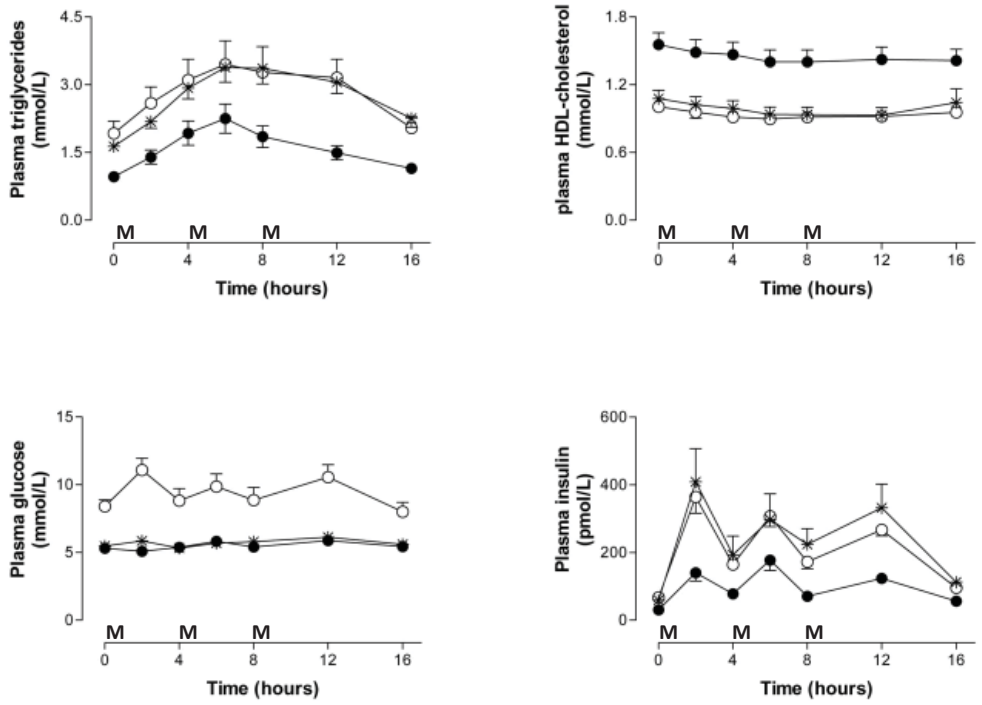

Figure 3.1 Time courses during 16-h of the postprandial response following 3 consecutive meals of plasma triglyceride, HDL-cholesterol, glucose, and insulin (mean ± SEM) in T2DM (open circles), MetS (asterisk) and healthy males (solid circles). Meal intake is indicated by M.

was similar in MetS and T2DM. Postprandially, plasma HDL-cholesterol decreased somewhat in all groups. In MetS and T2DM compared to the control group, AUCs for plasma triglycerides concentrations were higher, whereas AUCs for HDL-cholesterol were lower. Postprandial glucose increased in all groups at t=12-h, but a marked rise was observed only in T2DM. Insulin concentrations were elevated following each meal, reaching the highest concentrations after the third meal (t=12-h) in all groups, with the highest responses in MetS.

HDL and LDL were postprandially enriched in triglyceride-to-protein ratio in all three study groups (Figure 3.2) reaching a maximum concentration between 8 and 12 hours after breakfast. The lipoprotein triglyceride responses, expressed as AUC, were similar amongst the groups (Table 3.3). Postprandial HDL and LDL particles derived from T2DM and MetS versus controls had a decreased cholesterol content when expressed as AUC (Table 3.3), reaching the lowest values 8-h after breakfast (Figure 3.2). Postprandial

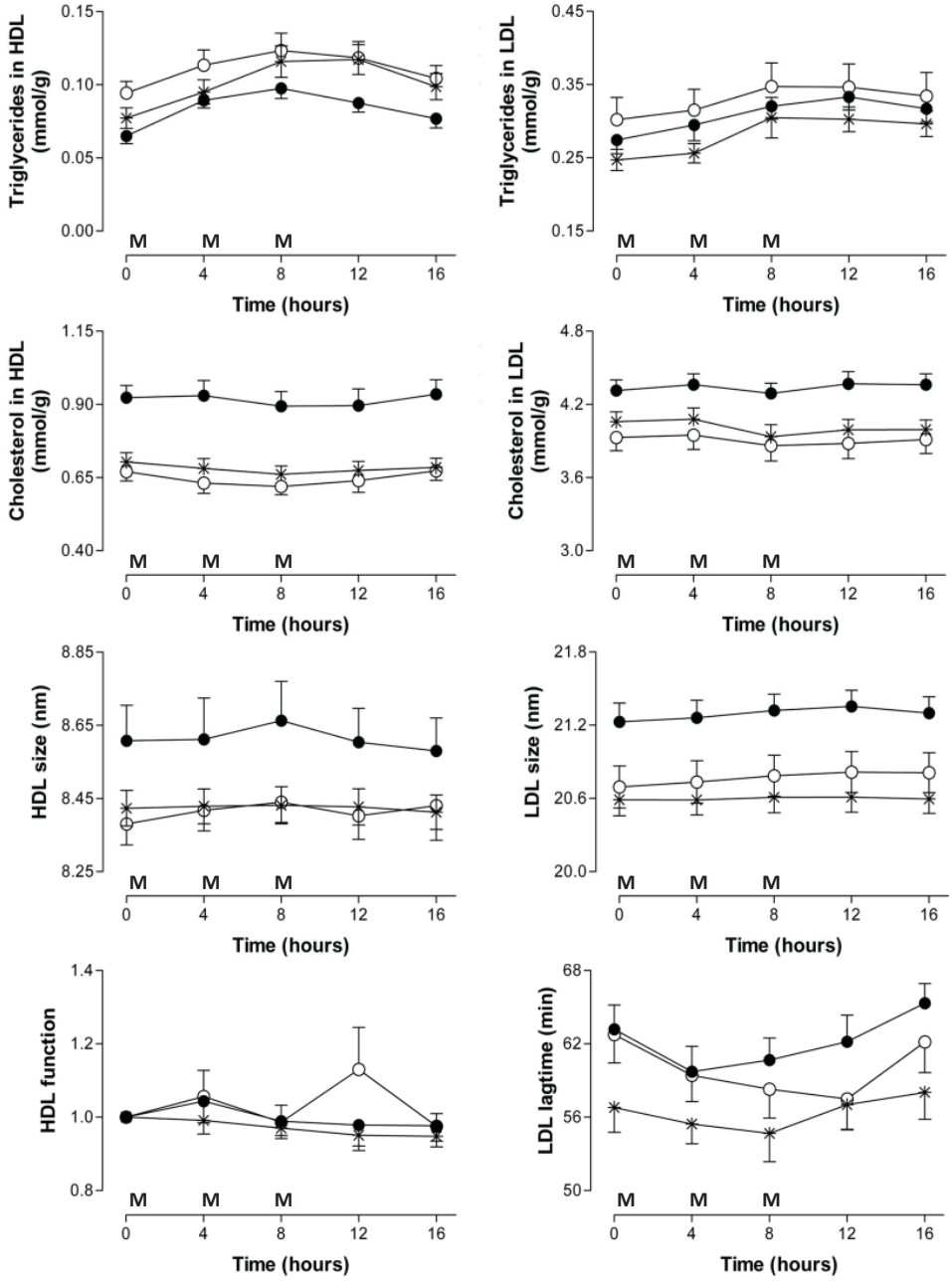

Figure 3.2 Time courses during 16-h of the postprandial response following 3 consecutive meals of high-density lipoprotein and low-density lipoprotein constituents (triglyceride and cholesterol) and size and of the anti inflammatory function of high-density lipoprotein and the susceptibility of low-density lipoprotein to oxidation (mean ± SEM) in T2DM (open circles), MetS (asterisk) and healthy males (solid circles). Meal intake is indicated by M.

HDL and LDL particles, especially those from T2DM, were larger compared to fasting diameters (Figure 3.2). In the T2DM group, the HDL size peaked at t=8-h and LDL at t=12-h. Postprandial changes in HDL size correlated significantly to changes in LDL size (r=0.47; P<0.01; all groups). The anti-oxidative capacity of HDL was hardly effected in the postprandial state, except for an change towards impairment after the third meal in T2DM (t=12-h; Figure 3.2), compared to both other groups. Subsequently, at the next time-point measured, HDL functionality returned to baseline level. The postprandial effect on HDL function was positively correlated to changes in HDL-cholesterol content and HDL particle size (r=0.50 and r=0.46, respectively; both P<0.01; all groups). LDL lag time was postprandially decreased in all groups after breakfast and subsequently returned toward baseline levels at t=4-h in controls, at t=8-h in MetS, and at t=12-h in T2DM. The most important correlate of postprandial changes in LDL lag time was the increase of plasma triglyceride concentration (r=0.29, P<0.05; all groups).

The P values of the time x group interaction terms for the physicochemical properties of LDL and HDL were all statistically non-significant (all P>0.05), except for the triglyceride to protein ration in HDL (P<0.05). However the differences of the AUC of the triglyceride to protein ration in HDL was non-significant (all P>0.05).

DISCUSSION

Our results show that plasma triglycerides increased significantly after breakfast and lunch, but remarkably no further increase was found after dinner. The prolonged hypertriglyceridemia following 3 consecutive meals in males with the MetS and patients with T2DM results in the formation of triglyceride-enriched HDL and LDL particles and an concomitant increased postprandial susceptibility of LDL to oxidation, especially in those with T2DM. Furthermore, enlarged lipoprotein particles and an impairment of the anti-oxidative capacity of HDL were postprandially observed in T2DM.

Insulin resistant states, including the MetS and T2DM, are associated with decreased plasma HDL-cholesterol concentration, whereas LDL-cholesterol concentration is usually normal. However, in T2DM the metabolism of LDL is modified, rendering the lipoprotein particles more atherogenic.[19] Furthermore, it has been shown that high-fat intake is associated with oxidative stress. For example, monocytes polymorphic nuclear cells release more superoxide anion when they are exposed to plasma from patients with hypertriglyceridemia.[20,21] In addition, whipping cream (75g of fat) resulted in an increase in nitrotyrosine, a marker of peroxinitrite and nitrosative stress generation, in normal subjects and T2DM patients.[22] The ensuing postprandial oxidative stress may trigger a number of atherogenic changes

including increases in inflammation, vasoconstriction and oxidation of LDL.[2] In the present study we have focused on (oxidative) modifications to both HDL and LDL, like composition, function, and particle size.

Lipoprotein particles have been demonstrated to be heterogeneous in size. Several studies measured LDL particle diameter using non-denaturing polyacrylamide gel electrophoresis in the postprandial state with inconsistent results. Small, but significant, reductions of LDL size of up to 0.1 nm were observed in myocardial infarction patients 6-h after an oral fat tolerance test,[23] and in apparently healthy men 4-h after a breakfast with high-fat.[24] A decrease in LDL size of 0.4 nm was observed in hypertriglyceridemic patients 6-h after an oral fat load.[25] The group of Taskinen measured LDL size 6-h after a high-fat meal in patients with coronary artery disease,[26] and 8-h after a mixed fat-rich meal in subjects with diabetes.[27] In both studies, LDL particles size remained statistically unchanged. In contrast to these studies, but in agreement to our study, a significant increase in LDL size (0.73 nm; $P<0.05$) was found in patients with primary mixed hyperlipidemia after an oral fat-tolerance test.[28] In the present study we used high-performance gel filtration chromatography for measurement of lipoprotein particle sizes, which is regarded as a very precise technique (CVs <0.2%),[16] and as such, small changes in diameter will be easily detected.

The mean size of HDL and LDL in T2DM was postprandially enlarged probably due to the increase in triglyceride content. Triglyceride in HDL and LDL is a good substrate for hepatic lipase, which hydrolyses triglycerides and thus ultimately converts lipoprotein particles smaller and denser. Since our follow-up duration was 16-h, it is possible that we have missed this latter part. Interestingly, HDL and LDL size peaked at different time points. In T2DM, HDL particle size peaked 8-h after breakfast and the largest mean LDL size was observed 4-h later, at t=12-h. These results suggest that the exchange in lipids, mediated by cholesterol-ester transfer protein (CETP), is faster in HDL than in LDL.

T2DM and MetS are associated with low-grade inflammation and oxidative stress. Therefore, it is likely that the antioxidant and anti-inflammatory properties of HDL, in which both the protein and lipid components are involved, are altered especially in postprandial conditions. Although the postprandial anti oxidative properties of HDL derived from T2DM was somewhat impaired after 3 consecutive meals at t=12-h, the current study observed no significant changes between- and within groups. The anti-inflammatory/oxidative properties of HDL in the postprandial state have been studied earlier in two relatively small studies with healthy subjects. Nicholls et al. showed that the anti-inflammatory potential of HDL was reduced after consumption of saturated fat and improved following the consumption of polyunsaturated fat.[29] Patel and coworkers demonstrated an impaired anti-inflammatory capacity of HDL in response to 20%

Intralipid.[30] In these studies, but in contrast to our study, the anti-inflammatory activity of HDL were measured using postprandial endothelial cells expression of intracellular cell adhesion molecules-1 (ICAM-1) and vascular cell adhesion molecules-1 (VCAM-1). It is currently not known whether this approach for HDL functionality is in agreement with the procedure that has been used in the present study.

LDL may become oxidized by an increase of free radical production (e.g. superoxide anion and hydroperoxyl radical) or nonradical oxidants (e.g. hydrogen peroxide and hypochorite). Oxidation of LDL is an important early event in the development of atherosclerosis. Oxidatively modified LDL is taken up by macrophages, which may become foam cells, leading to the formation of atherosclerotic plaques. In agreement with an earlier study,[17] fasting LDL lag times of T2DM and controls were not different, but interestingly the present study showed that postprandial LDL, especially derived from T2DM, is more susceptible to *in vitro* oxidation. Diwadkar et al. demonstrated that postprandial samples of diabetic subjects had a significantly shorter lag phase following one physiological meal (85g fat), but unfortunately they did not measure the lipid content of this lipoprotein class.[31] Of note, we demonstrated that the postprandial increase of LDL towards oxidation was positively associated with the triglyceride-to-protein ratio in LDL. In agreement, the highest triglyceride content in LDL and the most pronounced change in LDL oxidation has been found in T2DM.

There are several limitations of this study. In each group a relatively small number of subjects was examined and because our study has been conducted in men, the results may require replication in women. In addition, the exact peak of the highest postprandial concentrations is not known because a 4-h interval was used. Finally, we did not measure systemic oxidative stress levels.

Altogether, in subjects with the MetS and in patients with T2DM, the exaggerated and prolonged postprandial concentrations of plasma triglycerides lead to an increased triglyceride lipoprotein. The HDL cholesterol-to-protein ratio and HDL particle size have been shown to be important correlates of the anti oxidative properties of HDL, whereas the LDL triglyceride content was a determinant of LDL susceptibility to *in vitro* oxidation. In both fasting and postprandial samples, subjects with MetS and T2DM had smaller HDL and LDL particles. A prolonged period of increased LDL oxidisability was seen in T2DM. In conclusion, our study demonstrated that the exposure to 3 high-fat mixed consecutive meals results into pro-atherosclerotic changes of lipoprotein particles, especially in T2DM.

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5

Two consecutive high-fat meals affect endothelial-dependent vasodilation, oxidative stress and cellular microparticles in healthy men

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ABSTRACT

Background: A large body of evidence has accumulated indicating a relation between postprandial hyperglycemia and hypertriglyceridemia, and the risk of cardiovascular disease.

Objective: We studied possible mechanisms underlying the postprandial proatherogenic state by exposing healthy males to 2 consecutive high-fat mixed meals.

Patients/Methods: Seventeen healthy males (age 25.4±3 yrs, BMI 23.6±2 kg/m^2) were studied during 2 randomized visits. During the meal visit, subjects consumed standardized meals (50 g fat, 55 g carbohydrates, 30 g proteins) as breakfast and 4 hours later as lunch. During the control visit, subjects remained fasted. Prior to each blood collection (before and every 2 hours following the first meal), flow mediated dilatation (FMD) of the brachial artery was measured.

Results: Although within the normal range, postprandial plasma glucose and triacylglycerol concentrations increased significantly, especially after the second meal, as compared to baseline (4.8±0.3 to 5.4±0.4, 0.8±0.2 to 1.7±0.7 mmol/L, respectively; both $P<0.05$) and the fasting visit. Following the second meal, FMD was significantly impaired (6.9% versus 3.7%, $P<0.05$) whereas oxidized-LDL/LDL cholesterol ratio and malondialdehyde concentrations were markedly elevated (both $P<0.01$). Finally, an increase in total microparticle numbers was observed during the meal visit ($P<0.05$).

Conclusions: Mild elevations in plasma glucose and triacylglycerol in healthy males following 2 consecutive fat-rich meals were paralleled by impaired FMD, increased markers of oxidative stress and circulating microparticles, in particular, following the second meal. These findings may have consequences for subjects with postprandial dysmetabolism, including those with type 2 diabetes.

INTRODUCTION

In the late seventies of the previous century, Zilversmit postulated atherosclerosis to be a postprandial phenomenon.[1] Ever since, a large body of evidence has accumulated indicating a relation between postprandial hyperglycemia and hypertriglyceridemia, and the risk of cardiovascular disease (CVD).[2-7] The mechanisms that may link postprandial dysmetabolism to the increased CVD risk include postprandial endothelial dysfunction, as described in both healthy subjects and in patients at increased CVD risk, meal-induced oxidative stress, and inflammation.[7-10] Indeed, the observed correlations among postprandial triacylglycerol-rich-lipoproteins, endothelial dysfunction and selected oxidative stress markers suggest that triacylglycerols may reduce nitric oxide (NO) bioavailability by inducing oxidative stress.[8-10]

Oxidative stress is the common pathway by which many classic CVD risk factors adversely affect the vasculature and elevated levels of oxidative stress markers, such as oxidized low density lipoprotein (oxLDL), circulate in subjects at high risk of CVD.[11,12] Although prolonged exposure to dietary high fat increased plasma oxLDL concentrations,[13] no acute meal-induced elevations of oxLDL were reported in healthy subjects.

In addition to oxidative stress, the observed meal-related increases in circulating proinflammatory cytokines have been implicated in postprandial vascular dysfunction.[7,14,15] Some authors found postprandial elevations of proinflammatory molecules in patients with type 2 diabetes or postprandial leukocyte activation even in healthy subjects,[7,10] whereas others could not confirm these findings.[15,16]

Besides their previously reported procoagulant properties, adverse effects of cellular microparticles (MP) on endothelium-mediated vasodilation and their ability to induce cytokine production from endothelial cells *in vitro*, MP have recently also been suggested to reflect cellular stress.[17] Indeed, increased numbers of MP of various cellular origin are present in the circulation of patients at risk of CVD,[18-21] and the recently described meal-induced increase in circulating endothelial cell MP, in association with postprandial metabolic changes, seems in line with this hypothesis.[22] Thus, cellular MP may be one of the mechanisms linking postprandial metabolic and vascular functional derangements.

The main limitation of previous studies addressing postprandial dysmetabolism in relation to CVD risk was that only a *single* meal was given, while the subjects were fasted for the subsequent 8-10 hours. In fact, in many studies, artificial solid or liquid 'meals' were used which may not be compatible with the real-life situation. Thus, the metabolic responses following these artificial fat and/or glucose loads and the concomitant effects on the endothelium and blood cells elicited by the experimental condition may differ from

those occurring in real life. Therefore, in the present study, 17 healthy volunteers received 2 consecutive fat-rich mixed meals, as breakfast and lunch, respectively. In addition to the meal-related metabolic and endothelial functional responses, we measured markers of oxidative stress and inflammation, and cellular MP as possible mechanisms linking metabolic and vascular changes in the postprandial state.

SUBJECTS AND METHODS

Subjects

Seventeen healthy subjects were recruited from the VU University by billboard advertising and via advertisements at a local student-rowing club. Healthy males, Caucasian, aged between 20-35 years, having a body mass index (BMI) less than 27 kg/m^2 and a blood pressure <145/90 mmHg were eligible. Current smoking, the use of drugs (including anti-platelet drugs and NSAIDs) and the presence of CVD, hypertension, diabetes mellitus, or 1st degree family history of aforementioned diseases were exclusion criteria. Subjects were initially studied during a screening visit after giving written informed consent. The local ethics committee approved the study and the investigation conformed to the principles outlined in the Declaration of Helsinki.

Study design

Each subject was studied at 2 occasions, following an overnight fast (from 8.00 p.m. the previous evening). Subjects had to refrain from heavy physical activities during 24 hours prior to each visit. The order of the visits was randomized and the time interval between the control and meal visits was less than 4 weeks. During the meal day, the subjects received 2 consecutive, isocaloric (900 kcal) test meals at time point t=0 (breakfast) and 4 hours (lunch). Blood samples were collected before and at 2, 4, 6 and 8 hours following the first meal. Prior to each blood collection, vascular function was measured as FMD at the brachial artery (see below). During the control day, subjects were kept in the fasting state, only drinking water was allowed (maximum of 50 mL/hour to limit the effects on the autonomic nervous system due to gastric distension), and FMD and blood collection were performed similar to the meal day. Subjects remained in the supine position during both days in a quiet, temperature-controlled room.

Test meals

At the meal visit, each subject received 2 standardized fat-rich mixed-meals at breakfast (8.30 a.m.) and lunch-time (12.30 p.m.). Each meal consisted of 50 g fat, of which 60% was saturated, 55 g carbohydrates and 30 g protein. The breakfast consisted of EggMcMuffin® (McDonald's, affiliation Amsterdam-Sloten, The Netherlands), croissant with butter and marmalade, 200 mL of milk, combined with 20 ml of cream. The lunch consisted of Quarterpounder® (McDonald's, affiliation Amsterdam-Sloten, The Netherlands), croissant with butter, and 200 mL of milk. The subjects were instructed to consume each meal within 15 minutes.

Blood sample collection

To avoid endothelial and platelet activation artifacts, no in-dwelling canula was used and a new blood collecting system (Microflex, size 1.0 mm - 19 G, Vygon, France) was used for each blood collection. Venous blood was collected from the left antecubital vein at 15 minutes following each FMD measurement (which was invariably performed at the right arm), to avoid the effect of forearm occlusion on the parameters measured. The needle was placed at least 1 cm distal from the previous insertion and stasis was carefully avoided. Plasma was recovered after centrifugation (1550x*g*, 20 minutes, 20°C) and aliquots of 250 µL were snap frozen in liquid nitrogen within 30 minutes after withdrawal and stored at -80°C until assay. All samples from one subject were analyzed in the same series.

Endothelial function

Changes in brachial artery diameter in response to reactive hyperemia (FMD) were measured non-invasively using a high-resolution ultrasonic wall-tracking system (Wall-track System, PieMedical, Maastricht, The Netherlands). All individuals underwent the ultrasound examination according to the guidelines of the International Brachial Artery Reactivity Task Force.[23] In brief, the right brachial artery (20 mm proximal to the antecubital fossa) was visualized in B-mode using an 7.5 MHz linear probe (AU-5, Esaote, Maastricht, The Netherlands) and the baseline diameter was recorded. Then, during 5 minutes, a blood pressure cuff (Hokanson EC-4) around the forearm was inflated to 200 mmHg. After deflation of the cuff, the change in diameter was recorded every 30 seconds (up to 5 minutes after deflation). To avoid movement artifacts, we used a stereotactic probe-holding device with the subject's arm immobilized in a foam cast. Ten minutes after the final blood collection a second baseline recording was performed. Subsequently, 400 µg Nitroglycerin (NTG, Nitrolingual Spray, Pohl-Boskamp, Germany) was administered sublingually and the

change in brachial artery diameter was recorded at various time points (up to 10 minutes after NTG administration). Data were analyzed off-line using the Wall-track System. FMD and NTG-induced vasodilation were expressed as percentage change in diameter relative to baseline diameter. All measurements were performed by one experienced observer (MT). Reproducibility of the measurements showed a coefficient of variation <2%.

Biochemical measurements

Plasma glucose was measured by a hexokinase-based technique (Roche diagnostics, Mannheim, Germany), HbA_{1c} by HPLC. Insulin was measured by a commercially available immunoradiometric assay (Biosource/Medgenix Diagnostics, Fleurus, Belgium). Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triacylglycerols were determined by enzymatic methods (Modular, Hitachi, Japan). Low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula, insulin resistance according to the homeostasis model assessment of insulin resistance ($HOMA_{IR}$; (plasma glucose*insulin)/22.5). Non-esterified fatty acids (NEFA) were assessed by ELISA (WAKO chemicals, Neuss, Germany). OxLDL was measured by a competitive ELISA (Mercodia, Uppsala, Sweden), with intra-assay and inter-assay coefficients of variation of 4.8% and 7.8%, respectively. To obtain an estimate of the percentage of oxidized LDL particles, the oxLDL to LDL-C ratio (U/mmol) was calculated. Total malondialdehyde (MDA; μmol/L) was measured in EDTA-plasma, after reaction with thiobarbituric acid with an additional alkaline hydrolysis step as described previously.[24] The within run and between run variations were 3.5% and 8.7%, respectively. Both interleukin-6 (IL-6; pg/mL) and high-sensitive C-reactive protein (hs-CRP; mg/L) were measured in duplicate using ELISA (Diaclone, Besançon, France and Sanguin, Amsterdam, The Netherlands,[25] respectively).

Reagents and assays

All chemicals were of analytical quality. Phycoerythrin (PE)-labeled anti-glycophorin A (JC159, IgG_1) and anti-CD61-PE (Y2/51, IgG_1) were from Dako A/S (Glostrup, Denmark), anti-CD4-PE (CLB-T4/2,6D10, IgG_1) and anti-CD66e-PE (CLB-gran/10, IH4Fc, IgG_1) from the CLB (Amsterdam, The Netherlands), anti-CD8-PE (SK1, IgG_1), anti-CD14-PE (MØP9, IgG_{2b}), anti-CD20-PE (L27, IgG_1), IgG_1-PE (X40), IgG_{2b}-PE (S2), and IgG_1-peridinin chlorophyll-a protein (PerCP; X40) from Becton Dickinson (San Jose, CA). Anti-CD62e-PE (1.2B6, IgG_1) from Serotec Ltd (Oxford, England), anti-CD66b-PE (80H3, $IgG_{1\kappa}$) and fluorescein isothiocyanate (FITC)-labeled anti-CD66b (0531) from Coulter/Immunotech (Marseille, France). Allophycocyanin (APC)-labeled annexin V-APC from Pharmingen (San Jose, CA) and anti-CD106-FITC (B44498) was from Calbiochem-

Novabiochem Corporation (San Diego, CA). Anti-CD144-FITC (BMS 158FI) was from Bender MedSystems Diagnostics GmbH (Vienna, Austria).

Flowcytometric analysis of MP

MP were isolated as previously described.[19,20] The samples were analyzed in a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA). Fluorescence threshold were set using a calcium-containing buffer, annexin V and isotype-matched control antibody as described before.[19,20] MP were identified on forward scatter (FSC) and sideward scatter (SSC) characteristics, by binding of annexin V and a monoclonal antibody (MoAb) directed against a cell type-specific antigen. MP numbers were calculated as described earlier.[19,20]

Statistical analysis

Continuous variables are expressed as mean±SD, unless otherwise stated. To estimate the overall metabolic responses and the changes in MP during both visits, area under incremental curve (ΔAUC) of these parameters plotted against time were calculated. We used repeated-measures ANOVA, with time of measurement as the within factor and visit type as the grouping factor. Non-parametrical tests were performed for non-normally distributed data. Pair wise comparisons (Wilcoxon-Signed-Ranks tests) were applied as post hoc tests only when ANOVA revealed overall significant differences. Correlations were performed using the Spearman's test. A $P<0.05$ was considered statistically significant. Data were analyzed with SPSS for Windows, release 11.5.1.

RESULTS

The clinical and laboratory characteristics of the participants are depicted in Table 5.1. All participants were highly active, with an average physical training activity of 5.6±4 hours/week.

During the meal visit, plasma glucose, triacylglycerol and insulin concentrations rose significantly, especially after the second meal, as compared to baseline and the fasting visit (both $P<0.05$, $P<0.01$, $P<0.01$, respectively; Figure 5.1). Mean glucose concentrations rose from 4.8±0.3 to 5.4±0.4 mmol/L (t=6 hours, $P<0.001$ compared to baseline) and triacylglycerols increased from 0.8±0.2 to maximum mean values of 1.7±0.7 mmol/L (t=6 hours). Following the meals (t=2 and 6 hours), insulin concentrations increased to 75.2±32.2 and 114.2±37.6 pmol/L, respectively. HDL-C decreased from 1.37±0.2 to

Table 5.1 Baseline characteristics of the study population

	Healthy males (n=17)
Age, years	25.4±3
BMI, kg/m^2	23.6±1.8
Waist, cm	87.6±5
Systolic blood pressure, mmHg	116±8
Diastolic blood pressure, mmHg	75±7
HbA1c, %	5.1±0.2
Glucose, mmol/L	4.8±0.3
Insulin, pmol/L	33±10
$HOMA_{IR}$	1.0±0.3
Total-cholesterol, mmol/L	4.0±0.6
HDL-C, mmol/L	1.37±0.2
LDL-C, mmol/L	2.2±0.6
Triacylglycerols, mmol/L	0.8±0.3

Values are mean±SD. BMI indicates body mass index; $HOMA_{IR}$, homeostasis model assessment of insulin resistance; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol.

1.28±0.2 mmol/L (P<0.01) during the meal day (data not shown). During the control visit, glucose (from 4.9±0.3 to 4.6±0.3 mmol/L) and insulin (from 35.7±13.8 to 26.6±9.6 pmol/L) decreased significantly (both P<0.001 versus baseline). Triacylglycerol concentrations remained unchanged (Figure 5.1). Levels of NEFA remained low during the meal day with significant further lowering following both meals, suggesting insulin-mediated NEFA suppression (from 0.31±0.2 at baseline to 0.13±0.1 at t=2 hours after the first meal; and from 0.28±0.1 prior to the second meal to 0.20±0.1 mmol/L at t=2 hours after the second meal, respectively, both P<0.001). As expected, NEFA concentrations rose significantly during the fasting day from 0.32±0.1 to 0.78±0.2 mmol/L (at t=8 hours; P<0.001 versus baseline and meal day; Figure 5.1).

FMD showed a marked reduction following the second meal (6.9% versus 3.7%; P<0.05) as compared to baseline (Figure 5.2). The difference between FMD at t=6 hours between both days tended to be statistically significant (P=0.051). NTG-induced vasodilation measured at the end of both days did not differ (both 13.0%). Changes in FMD showed no significant correlation with the metabolic changes or MP numbers (r=-0.36, P=0.31, for FMD and total MP).

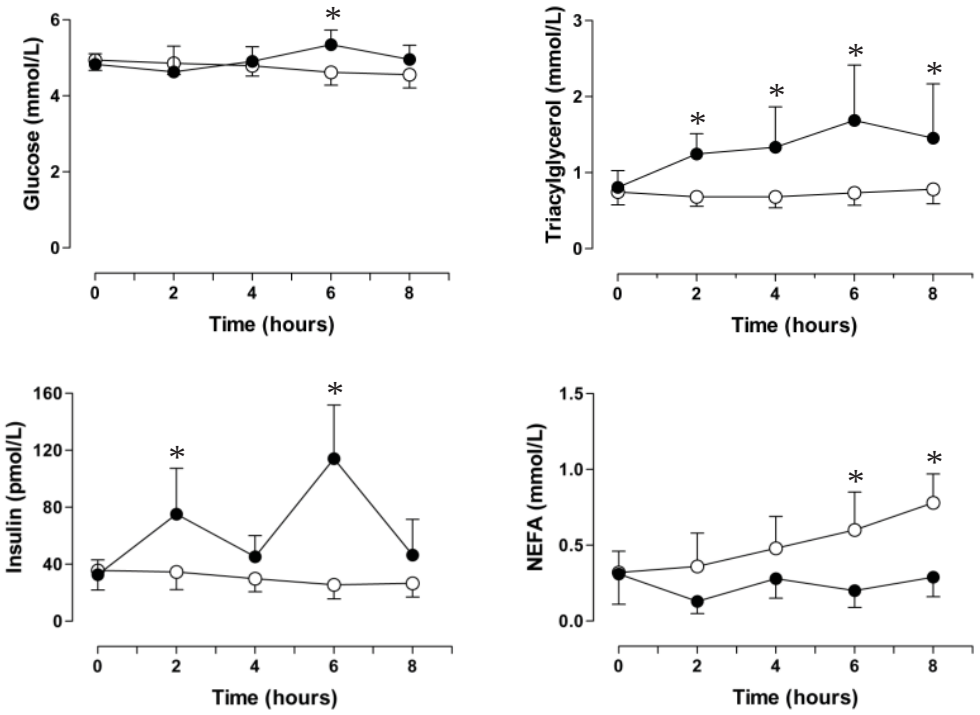

Figure 5.1 Plasma concentrations of glucose, insulin, triacylglycerols and NEFA during the meal- (solid circles) and fasting days (open circles) in healthy lean subjects. Data are mean±SD. *P<0.05.

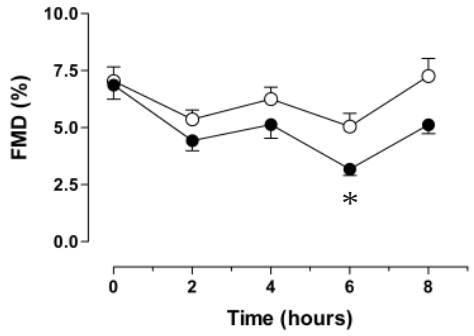

Figure 5.2 Changes in flow-mediated dilation (FMD) during the meal- (solid circles) and fasting days (open circles) in healthy lean subjects. Data are mean±SEM. *P<0.05.

OxLDL and MDA concentrations were measured at baseline (t=0) and t=6 hours during both visits (i.e. 6 hours after the first meal on the meal day, and 6 hours after the first blood collection during the fasting visit). During the meal visit, the oxLDL/LDL-C ratio increased from 19.4±2.8 to 22.4±4.5 U/mmol (P=0.001 compared to baseline and fasting day; Figure 5.3). During the fasting visit, oxLDL/LDL-C remained constant (18.7±2.8 and 18.1±2.3 U/mmol). Postprandial changes in oxLDL/LDL-C correlated with the changes in triacylglycerol levels (r=0.60, P=0.01) but not with changes in glucose concentrations. Similarly, MDA increased during the meal day from 7.2±0.6 to 7.8±0.8 μmol/L and decreased during the fasting day from 7.8±0.7 to 7.0±0.5 μmol/L (P=0.01 and P=0.001, respectively and P=0.001 meals versus fasting; Figure 5.3). Changes in MDA correlated with meal-induced changes in glucose concentrations (r=0.67, P=0.003) but not with increases in postprandial triacylglycerols. Postprandial MDA elevations tended to correlate inversely with FMD changes (r=-0.52, P=0.059; Figure 5.4).

Circulating numbers of MP from T helper, T suppressor, B cells, granulocytes, and endothelial cells were negligible (data not shown). Therefore, we only report MP from platelets, erythrocytes and monocytes, in addition to the total MP count. The total numbers of MP, of which the largest fraction is platelet-derived (88-98%), increased during the meal visit compared to the fasting visit (P<0.05; Figure 5.5A and 5.5B). The overall changes in platelet-derived MP (PMP), calculated as ΔAUC PMP, correlated with the percentile change in oxLDL/LDL during the meal day (r=0.59, P=0.042). The fraction of erythrocyte-derived MP, comprising 2-3% of total MP numbers during fasting, increased to 3-11% after

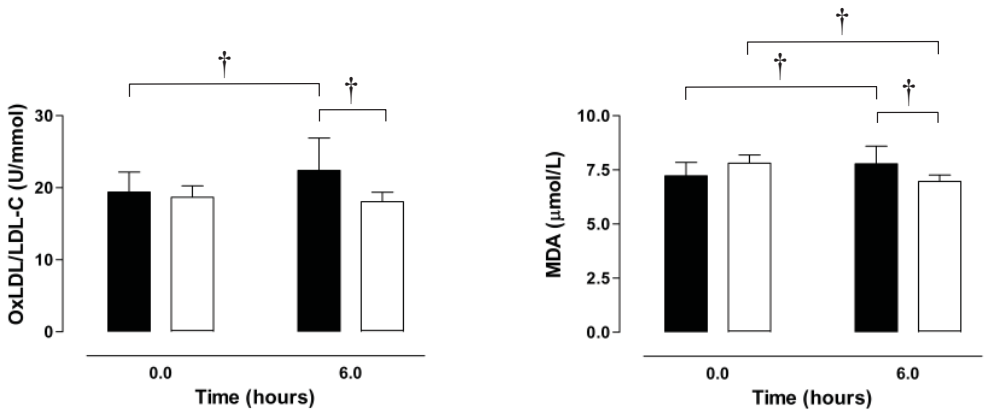

Figure 5.3 OxLDL/LDL-C ratios and malondialdehyde (MDA) concentrations at baseline (t=0 hours) and at t=6 hours during the meal- (black bars) and fasting (white bars) days. Data are mean±SD. †P<0.01.

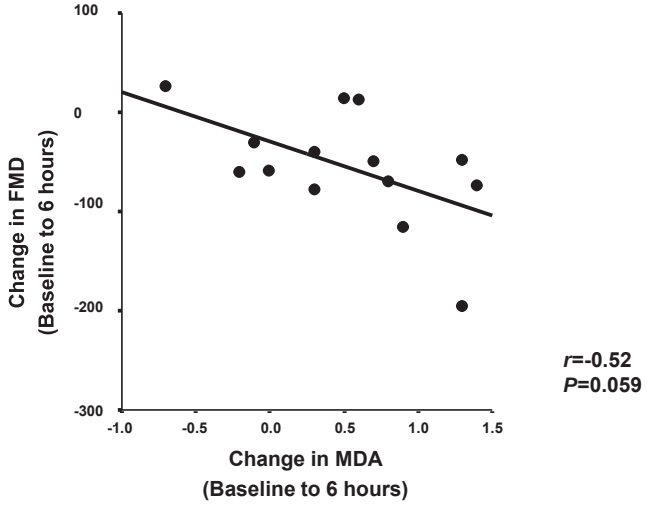

Figure 5.4 Scatter plot representing the relationship between postprandial percentual changes in flow mediated dilatation (FMD) and absolute changes in malondialdehyde (MDA) concentrations.

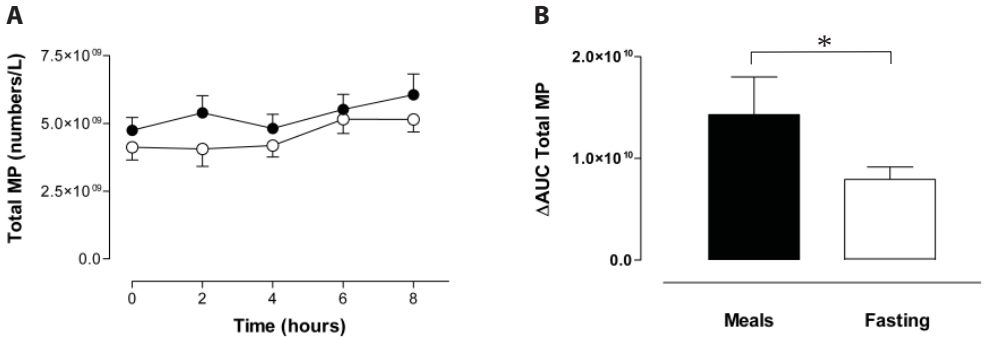

Figure 5.5 Courses of plasma total microparticles (MP) concentrations during the meal (solid circles) and fasting (open circles) visit (A). The area under the incremental curve (ΔAUC) of total microparticle (MP) numbers during the meal visit (black bars) as compared to the fasting visit (white bars) (B). Data are mean±SEM. *P<0.05.

consumption of both meals (P<0.001, versus baseline and fasting day). Finally, monocyte-derived MP, constituting 0.3% of the total MP numbers, tended to increase during the meal visit only (P=0.07 versus baseline; data not shown).

Plasma IL-6 concentrations followed an identical "U-shaped" curve on both days, with the lowest values after noon (t=4 hours after baseline, P<0.03). CRP remained unchanged during both days (Figure 5.6).

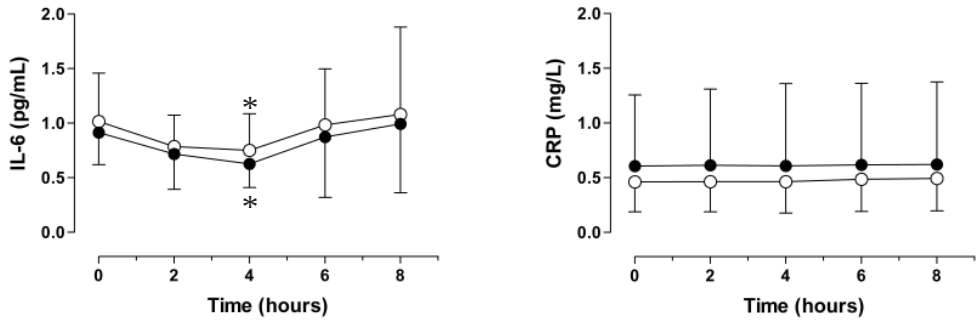

Figure 5.6 The course of plasma interleukin-6 (IL-6) and C-reactive protein (CRP) concentrations during the meal- (solid circles) and fasting (open circles) days in healthy lean subjects. Data are mean±SD. *P<0.05.

DISCUSSION

The present study demonstrates that exposure of healthy, physically active, lean and insulin sensitive young males to two consecutive fat-rich mixed meals results in impaired endothelium-dependent vasodilatation and acute elevation of oxLDL and MDA concentrations despite relatively mild metabolic changes. All postprandial changes were most marked following the second meal, and the meal-related increase in oxidative stress associated with postprandially elevated numbers of circulating PMP.

Both *in vivo* and *in vitro* studies indicate that (postprandial) hypertriglyceridemia adversely affects several endothelial functions, including exposure of adhesion molecules and NO-mediated vasodilatation by inducing oxidative stress.[7-9,26,27] In healthy subjects, postprandially impaired FMD correlated with meal-induced hypertriglyceridemia in some,[9,28,29] but not in other studies.[8,10] In our study, the decrease in FMD following the second meal was not correlated to the concomitant plasma triacylglycerol elevations, but rather tended to associate with the meal-related increase in levels of oxidative stress markers. There are several possible explanations for these seemingly discrepant observations. Firstly, the populations studied are not readily comparable (i.e. very physical active and therefore more insulin sensitive compared to persons with a more sedentary lifestyle), secondly, the postprandial metabolic changes found in our subjects were relatively mild, as compared to those observed in other studies,[9,14,29] thirdly, the composition and consistency (liquid versus solid) of the test-meals differed in the various studies, and finally, the changes in the different parameters measured, in relation to time following meal ingestion, cannot easily be established.

We found lower baseline concentrations of oxLDL/LDL-C (19.4 U/mmol) compared to findings by others who reported oxLDL/LDL-C concentrations of 21.5 and 23.6 U/mmol in elderly control subjects and type 2 diabetic patients, respectively.[12] These authors found a correlation between fasting plasma triacylglycerols and oxLDL/LDL-C.[12] Our results extend these observations to the postprandial state. Although alterations in plasma oxLDL concentration by dietary changes have been described previously,[13] these changes occurred only after exposure to a low vegetable diet for several weeks, rather than acutely in response to a meal. Previously, it was suggested that oxLDL can promote the shedding of PMP *in vitro*.[21] Our present study shows that this association may also exist *in vivo*. In addition, we found a postprandial increase in plasma MDA concentrations, also suggesting oxidative stress, and this was associated with FMD.

In contrast to Ferreira,[22] we could not identify any MP from endothelial cells. A possible explanation is that we only used endothelial cell-specific antibodies (CD62e, CD106 and CD144), and that the postprandial increases in CD31-positive MP reported by these authors may be in part due to elevated PMP.[30,31]

Because PMP have often been associated with procoagulant activity,[18,19] we determined prothrombin fragment F_{1+2} and thrombin antithrombin complexes. However, no difference was found in these coagulation parameters between fasting and meal days (data not shown). PMPs, in addition to their procoagulant potential, were previously shown to also posses anti-coagulant activity.[19] Thus, the simultaneous presence of these opposing properties may overall have resulted in no measurable effect on thrombin generation in our study.

The lack of association between MP numbers and changes in FMD during meal days does not preclude that MP may directly impair NO-bioavailability *in vivo*, as was observed *in vitro*,[32] since the association between circulating MP, representing the result of MP formation and clearance, and NO-mediated vasodilation may be difficult to assess due to the dynamic characteristics of both variables. However, the observed association between MP and oxLDL suggest that MP, albeit indirectly, may play a role in postprandial endothelium-dependent vasodilatation.

Despite previously reported plasma IL-6 elevations following a meal and its association with postprandial hypertriglyceridemia,[7,14] we found no postprandial changes in IL-6 concentrations. Interestingly, similar to others, we found IL-6 tend to display a circadian rhythm.[33] In accordance to Tsai et al., who gave one high-fat meal to healthy males, postprandial CRP remained unchanged in our subjects.[10] Thus, in healthy, lean and insulin-sensitive subjects, the postprandial metabolic changes do not elicit acute proinflammatory effects.

The present study suggests that even in young healthy subjects high-fat diets may be atherogenic due to oxidative stress-induced impairment of endothelium-dependent vasodilatation. We suggest that, in order to obtain a reliable impression of the total exposure of the vasculature to meal-related derangements, postprandial studies should use a real-life approach by giving several (consecutive) test meals, at regular intervals, during one day. Our findings may have consequences for insulin resistant and type 2 diabetic patients, who suffer from an impaired clearance and suppression of postprandial triacylglycerol-rich lipoproteins resulting in prolonged and exaggerated postprandial metabolic abnormalities.

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6

Postprandial changes in the phospholipid composition of circulating microparticles are not associated with coagulation activation

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ABSTRACT

Evidence is present that the phospholipid composition of circulating cell-derived microparticles (MP) affects coagulation *in vivo*, and that postprandial metabolic alterations may be associated with hypercoagulable state. Our objective was to investigate whether postprandial metabolic responses affect the phospholipid composition of MP, and whether such changes are associated with coagulation activation. Therefore, 12 healthy males were studied twice and randomly received 2 consecutive meals or remained fasted. Blood was collected before and at 2, 4, 6 and 8h following breakfast. The phospholipid composition of MP was determined by hpTLC. Numbers and cellular origin of MP were determined by flowcytometry. Plasma concentrations of prothrombin F_{1+2} and thrombin-antithrombin-complexes were measured. The *in vitro* procoagulant activity of MP was studied by fibrin generation. During the meal visit, plasma glucose, triglyceride and insulin levels increased, compared to baseline and the fasting visit (all $P<0.05$). Postprandially, the total numbers of MP increased in time compared to the fasting visit ($P<0.05$). Especially erythrocyte-derived MP increased (6-fold) during the meal visit, but remained constant on the fasting day ($P<0.001$). On the meal versus fasting day circulating MP contained increased phosphatidylcholine ($P<0.05$) and decreased sphingomyelin ($P<0.05$) amounts. The amount of phosphatidylserine did not change. Concentrations of plasma F_{1+2} and thrombin-antithrombin were similar on both days, as was the ability of MP to generate fibrin *in vitro*. Although the numbers, cellular origin and phospholipid composition of MP change during exposure to 2 consecutive meals in healthy subjects, this does not lead to changes in the coagulation activation *in vivo*.

INTRODUCTION

Clinical and epidemiological evidence indicates that postprandial elevations of plasma glucose and triglyceride-rich lipoproteins are related to the risk of atherothrombotic disease.[1] To which extent postprandial coagulation activation contributes to this risk is still under debate. Several investigators reported postprandial coagulation activation in healthy subjects and in patients suffering from cardiovascular disease, as measured by increased plasma concentrations of factor VIIa, prothrombin fragment F1+2 (F_{1+2}) or thrombin-antihrombin complexes (TAT).[2-11] Explanations for the underlying mechanism(s) of coagulation activation in the postprandial state, however, range from activation of factor VII (FVII) and XI (FXI) by triglycerides to increased activity of plasminogen activator inhibitor (PAI). Of interest, other investigators found that postprandial FVII activation was not associated with increased concentrations of F_{1+2} and TAT.[12,13] Similarly, there is no consensus among investigators whether platelet activation occurs in the context of postprandial lipemia.[14,15]

By exposing negatively charged phospholipids, mainly phosphatidylserine, but also by exposing tissue factor, cell-derived microparticles (MP) can propagate and even initiate coagulation.[16-18] Therefore, MP have been associated with coagulation activation in health and disease, and elevated numbers of circulating MP are related with an increased risk of thromboembolic events as well as with disseminated intravascular coagulation.[16-18] Recently, we demonstrated that the total number of MP in plasma from healthy males is elevated following consecutive meals, suggesting that the postprandial state induces the formation of MP.[19] At present, it is unknown to which extent quantitative and qualitative characteristics of MP are associated with coagulation in the postprandial state. Therefore, we investigated whether the postprandial state affects not only the numbers but also the cellular origin and phospholipid composition of circulating MP, their procoagulant phenotype and the association with coagulation activation *in vivo*.

MATERIALS AND METHODS

Subjects

Twelve healthy males, Caucasian, aged between 20-35 years, having a body mass index (BMI) less than 27 kg/m^2 and a blood pressure <145/90 mmHg were included as previously described.[19] Current smoking, the use of drugs (except for incidental analgesic agents) and the presence of CVD, hypertension, coagulation disorders, diabetes mellitus, or 1st degree family history of aforementioned diseases were exclusion criteria. Subjects were initially

studied during a screening visit after having given written informed consent. The local ethics committee approved the study and the investigation conformed to the principles outlined in the Declaration of Helsinki.

Study design

Each participant was studied at 2 occasions, following an overnight fast (from 8.00 PM the previous evening). Subjects had to refrain from heavy physical activities during 24 hours prior to each visit. During the meal day, the participants received 2 consecutive, isocaloric (900 kcal) test meals at time points t=0 (breakfast) and 4 hours (lunch). Blood samples were collected before and at 2, 4, 6 and 8 hours following the first meal. During the fasting day, blood samples were collected at similar times from 8.00 AM onwards and the subjects were only allowed water at libitum. The order of the visits was randomized and the time interval between the control and meal visits was less than 4 weeks.

Test meals

At the meal visit, each subject received 2 standardized fat-rich mixed-meals at breakfast (8.30 AM) and lunch-time (12.30 PM). Each meal consisted of 50 g fat, of which 60% was saturated, 55 g carbohydrates and 30 g protein. The breakfast consisted of EggMcMuffin® (McDonald's, affiliation Amsterdam-Sloten, The Netherlands), croissant with butter and marmalade, 200 mL of milk, combined with 20 ml of cream. The lunch consisted of Quarterpounder® (McDonald's, affiliation Amsterdam-Sloten, The Netherlands), croissant with butter, and 200 mL of milk. The participants were instructed to consume each meal within 15 minutes.

Blood sample collection

To avoid coagulation, endothelial, and platelet activation artifacts, no in-dwelling canula was used and a new blood collecting system (Microflex, size 1.0 mm - 19 G, Vygon, France) was used for each blood collection. Venous blood was collected from the left antecubital vein, the needle was placed at least 1 cm distal from the previous insertion and stasis was carefully avoided, into 0.1 volume of 105 mmol/L trisodium citrate. Plasma was recovered after centrifugation (1550*g*, 20 minutes, 20°C) and aliquots of 250 μL were snap frozen in liquid nitrogen within 30 minutes after withdrawal and stored at -80°C until assay. All samples from one subject were analyzed in the same series.

Biochemical measurements

Plasma glucose was measured by a hexokinase-based technique (Roche diagnostics, Mannheim, Germany), HbA_{1c} by HPLC. Insulin was measured by a commercially available immunoradiometric assay (Biosource/Medgenix Diagnostics, Fleurus, Belgium). Plasma total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides were determined by enzymatic methods (Modular, Hitachi, Japan). Low-density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula. Non-esterified fatty acids (NEFA) were assessed with an enzymatic colorimetric method (WAKO chemicals, Neuss, Germany). F_{1+2} and TAT were determined by ELISA (Dade Behring, Eschborn, Germany).

Reagents and assays

Phospholipid standards were obtained from Larodan (Malmö, Sweden): l-α-phosphatidylcholine, l-α-lyso-phosphatidylcholine, sphingomyelin, l-α-phosphatidylserine, l-α-phosphatidylinositol, l-α-phosphatidylethanolamine. The l-α-lyso-phosphatidylethanolamine standard was obtained from Sigma (St. Louis, MO). Chloroform (analytical grade) and ethylacetate, acetone, methanol, ethanol, dichloromethane, isopropanol, acetic acid (all HPLC grade) and HPTLC plates (Cat. No. 1.05641; 20 x 10 cm, Silicagel 60Å pore size, particle size 5-17 μm, 0.2 mm layer thickness) were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical quality. Phycoerythrin (PE)-labelled anti-glycophorin A (JC159, IgG_1) and anti-CD61-PE (Y2/51, IgG_1) were from Dako A/S (Glostrup, Denmark), anti-CD4-PE (CLB-T4/2,6D10, IgG_1), anti-CD66e-PE (CLB-gran/10, IH4Fc, IgG_1) from Sanquin (Amsterdam, The Netherlands), anti-CD8-PE (SK1, IgG_1), anti-CD14-PE (MØP9, IgG_{2b}), anti-CD20-PE (L27, IgG_1), IgG_1-PE (X40), IgG_{2b}-PE (S2) and IgG_1-peridinin chlorophyll-a protein (PerCP; X40) from Becton Dickinson (San Jose, CA), anti-CD62e-PE (1.2B6, IgG_1) from Serotec Ltd (Oxford, England), anti-CD66b-PE (80H3, $IgG_{1\kappa}$) and fluoresceinisothiocyanate (FITC)-labeled anti-CD66b (0531) from Coulter/Immunotech (Marseille, France), and allophycocyanin (APC)-labelled annexin V-APC from Pharmingen (San Jose, CA), anti-CD106-FITC (B44498) from Calbiochem-Novabiochem Corporation (San Diego, CA) and anti-CD144-FITC (BMS 158FI) from Bender MedSystems Diagnostics GmbH (Vienna, Austria).

Isolation of MP

Per time point, two 250 μl aliquots were thawed on melting ice, and centrifuged for 30 minutes at 17,590*g* and 20°C. The supernatant (225 μl) was removed and 225 μl phosphate-buffered saline (PBS) containing 0.32% trisodium citrate (154 mmol/L NaCl,

1.4 mmol/L phosphate and 10.9 mmol/L trisodiumcitrate, pH 7.4) was added. The MP were resuspended and again centrifuged for 30 minutes at 17,590g and 20°C. For flowcytometry, 225 µl supernatant was removed. Subsequently the MP were resuspended and diluted 4-fold with PBS/citrate buffer, of which 5 µl was used per incubation. For Bligh & Dyer extraction, the supernatant (225 µl) was removed and the pellet was washed once more. Finally, 225 µl supernatant was removed and the MP were resuspended in the remaining 25 µl.

Flowcytometric analysis of MP

The samples were analyzed in a FACScan flowcytometer with CellQuest software (Becton Dickinson, San Jose, CA). Forward scatter (FSC) and sideward scatter (SSC) were set at logarithmic gain. To distinguish MP from events due to noise, MP were identified not only on FCS and SSC characteristics, but also by binding of annexin V and a MoAb directed against a celltype-specific antigen. To identify annexin V-positive MP, a threshold was placed in a MP sample prepared in the presence of annexin V without calcium to correct for autofluorescence. To identify MP that bind cell type-specific MoAbs, the MP were incubated with identical concentrations of isotype-matched control antibodies to set the threshold. FITC-fluorescence was measured in the FL-1 channel and PE-fluorescence in the FL-2 channel.

To 35 µl of PBS/calcium buffer (154 mmol/L NaCl, 1.4 mmol/L phosphate and 2.5 mmol/L $CaCl_2$, pH 7.4), 5 µl annexin V-PE and 5 µl FITC-labeled cell-specific MoAbs or isotype-matched control antibody (IgG_1-FITC) were added. MP (5 µl) were added, and the mixtures were incubated in the dark for 15 min at room temperature. For the CD66e-PE analysis an IgG_1-PE control antibody was used. Subsequently, 900 µl PBS/calcium buffer was added to stop the labeling. All samples were analyzed for 1 minute during which the flowcytometer analyzed about 150 µl of the MP suspension. From the number of events (N_x) in the upper right (marker and annexin V-positive) quadrant of the flowcytometric analysis (FL-1 versus FL-2 corrected for isotype control and autofluorescence), the number of MP/L plasma was calculated: $Number/L=N_x[100/5]x[950/60]x[10^6/250]$, in which 5 (µL) is the volume of MP suspension, 100 is the total volume of washed MP suspension, 950 is the total volume in the tube before analysis, 60 is the sample volume analyzed, 10^6 is the number of µL/L, and 250 is the original volume of plasma.

Phospholipid extraction

Phospholipids were extracted according to Bligh & Dyer.[20,21] To improve the recovery for phosphatidylserine and phosphatidylinositol, the water phase was replaced by acetic acid (0.5%).

For each time point the remaining 25 µl of the two 250 µl aliquots were pooled. To these 50 µl samples, 3 ml methanol:chloroform (2:1) was added. The samples were thoroughly mixed for 30 s and 750 µl 0.5% acetic acid was added. Again the samples were mixed for 30 s. Then, chloroform (1 ml) and 0.5% acetic acid (800 µl) were added. After each addition, the samples were mixed for 30 s. Subsequently the samples were centrifuged for 10 minutes at 1560g (20°C). The chloroform fraction (at the bottom) was isolated, and the aqueous fraction was washed twice with chloroform (1 mL). The three chloroform fractions were pooled and dried under nitrogen at 40°C. The samples were stored overnight in -20°C, and redissolved in methanol:chloroform (2:1) to be applied to hpTLC plates.

High performance Thin Layer Chromatography (hpTLC)

To analyze cholesterol and phospholipid contents of the samples, separate hpTLC plates were used, because the developing solvent mixtures differ as previously described.[21] All hpTLC plates were prerun (full height) with methanol:ethylacetate (6:4) in a Camag horizontal developing chamber (Merck) to remove impurities. The plates were activated for 10 minutes at 130°C. The first plate was used for phospholipids analysis, whereas the second plate was used for cholesterol analysis.

The identification of phospholipids species was based upon a standard mixture of commercial phospholipid standards that was applied to each hpTLC plate. The density of these spots was analyzed by photodensitometric scanning by a GS-800 Calibrated Imaging Densitometer (Bio-Rad; Veenendaal, The Netherlands), quantified using Quantity One software version 4.2.2 (Bio-Rad; Veenendaal, The Netherlands) and expressed as arbitrary units.

Since plasma contains (phospho-)lipids and cholesterol, especially in the postprandial state, and the (washed) MP fraction still contains 1% plasma (final concentration), the lipid composition of MP was corrected for this confounder. Therefore, 25 µl of supernatant from the first washing step was taken and 225 µl of PBS containing 0.32% trisodiumcitrate (pH 7.4) added. These samples were also washed twice, extracted and analyzed, exactly as described for the MP preparation.

Fibrin generation test (FGT)

The ability of MP to generate fibrin was measured directly in plasma of the participants in the absence or presence of anti-human (coagulation) factor VII, anti-human factor XI or anti-human factor XII (all provided by Sanquin, Amsterdam, The Netherlands) as previously described.[22] After pre-incubation for 5 minutes at 37°C, clotting of plasma was initiated by addition of $CaCl_2$. Fibrin formation was determined by measuring the optical density (λ=405 nm) in duplicate on a spectrophotometer (SPECTRAmax microplate reader; Molecular Devices Corp., Sunnyvale, CA) at 37°C.

Statistical analysis

Continuous variables are expressed as mean±SE, unless otherwise stated. To estimate the overall metabolic responses and the changes in MP during both visits, area under the curve (AUC) of these parameters plotted against time were calculated. Non-parametrical tests were performed for non-normally distributed data. Correlations were performed using the Spearman's test. A $P<0.05$ was considered statistically significant. Data were analyzed with SPSS for Windows, release 15.0.

Table 6.1 Baseline and fasting characteristics of the study population

	Healthy males (n=12)
Age, yrs	26.0±0.9
BMI, kg/m^2	23.9±0.4
Waist, cm	87.6±1.3
Systolic blood pressure, mmHg	116±2
Diastolic blood pressure, mmHg	72±2
HbA1c, %	5.2±0.1
Glucose, mmol/L	4.9±0.1
Insulin, pmol/L	34±3
Total-cholesterol, mmol/L	4.0±0.2
HDL-C, mmol/L	1.36±0.1
LDL-C, mmol/L	2.3±0.2
Triglycerides, mmol/L	0.8±0.1

Values are mean±SE. BMI indicates body mass index; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol.

RESULTS

Postprandial metabolic changes

The clinical and baseline laboratory characteristics of the participants are depicted in Table 6.1. The baseline concentrations of all parameters did not differ between the two study days. During the meal visit, plasma glucose, triglyceride and insulin concentrations rose significantly, especially after the second meal, albeit all within the normal range, as compared to baseline and the fasting visit (both $P<0.05$, $P<0.001$, $P<0.001$, respectively; Figure 6.1). During the fasting visit, plasma concentrations of NEFA rose significantly from 0.29±0.1 to 0.74±0.1 mmol/L ($P<0.001$ versus baseline and meals day).

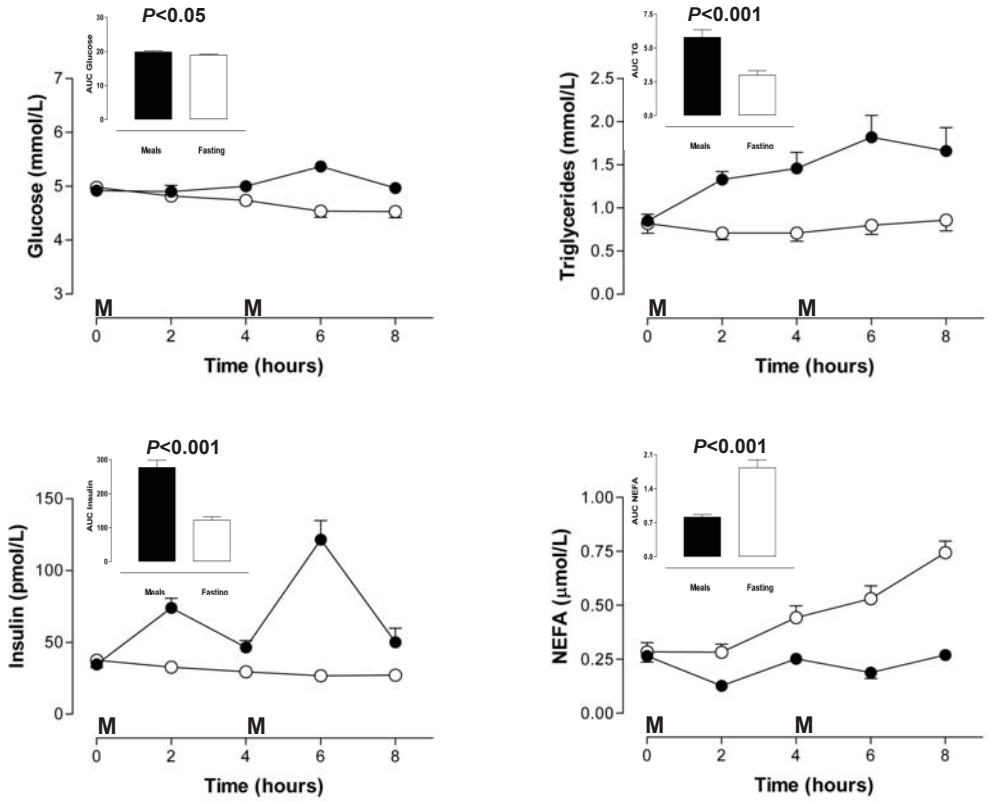

Figure 6.1 Plasma concentrations of glucose, triglycerides, insulin and NEFA during the meal (solid circles) and fasting days (open circles) in healthy males. Meal intake is indicated by M. Bars (black, meals; white, fasting) in the insets represent respective AUC values. The *P* value is given for AUC difference. Data are mean±SE.

Postprandial changes in circulating MP

The total numbers of MP, of which the largest fraction is platelet-derived (88-98%), increased during the meal visit compared to the fasting visit ($P<0.05$; Figure 6.2). Mean concentrations of erythrocyte-derived MP increased to more than 6-fold after consumption of both meals ($P<0.001$, versus baseline and fasting day), and remained unaffected during the fasting day. Finally, monocyte-derived MP concentrations, constituting only 0.3% of the total numbers of circulating MP, slightly increased during the meal visit ($P=0.07$ versus fasting). MP from lymphocytes, granulocytes, and endothelial cells were not detectable. The numbers of erythrocyte-derived MP correlated significantly with the corresponding insulin concentrations measured on both days ($r=0.65$, $P<0.001$).

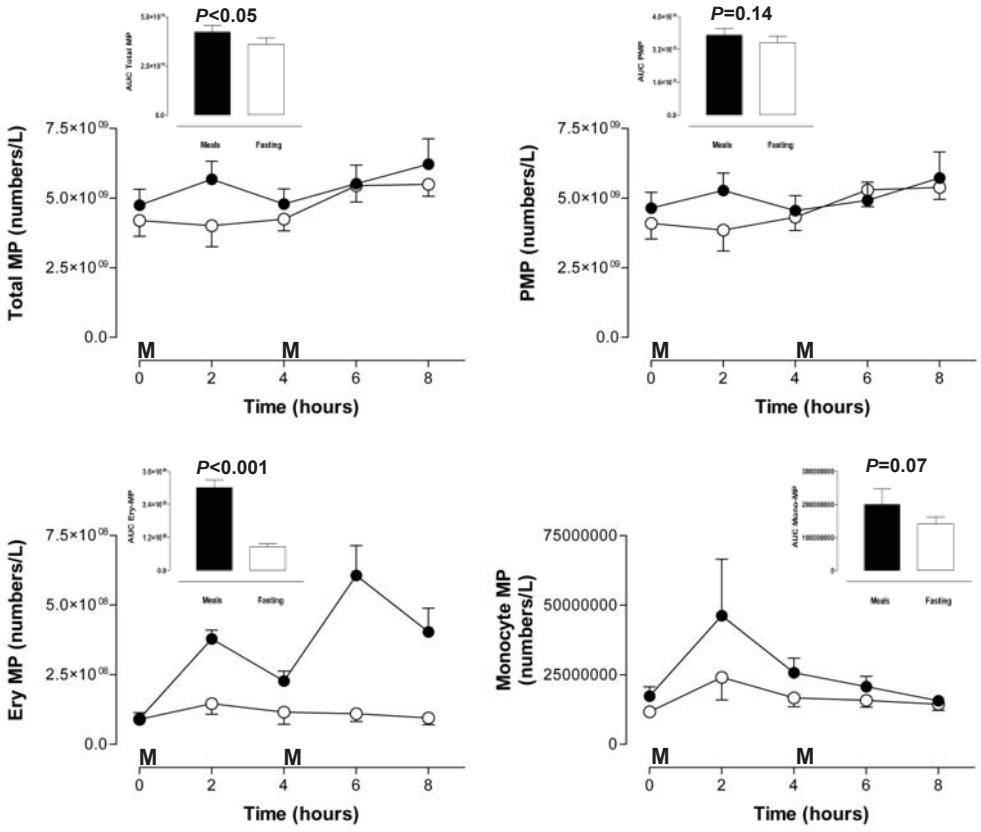

Figure 6.2 The course of total MP, platelet-derived MP, erythrocyte-derived MP and monocyte-derived MP numbers/L during the meal (solid circles) and fasting (open circles) days in healthy males. Meal intake is indicated by M. Bars (black, meals; white, fasting) in the insets represent respective AUC values. The *P* value is given for AUC difference. Data are mean±SE.

Postprandial changes in the phospholipid composition of circulating MP

Table 6.2 summarizes the overall phospholipid compositions of MP during the meal (+) and fasting (-) day, expressed as percentage of total phospholipid (mol/mol) after correction for the amount of phospholipids in MP-free plasma (see Methods). Compared to the fasting day, the relative % of phosphatidylcholine increased, whereas concurrently sphingomyelin decreased. Furthermore, the amount of phosphatidylinositol increased following the first meal. The phospholipids phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine were not detectable in plasma that had been depleted from MP.

The cholesterol : phospholipid ratio of MP during the meals day did not change significantly. During the fasting day, however, the cholesterol : phospholipid ratio increased significantly from 0.34±0.1 to 0.49±0.1 (P<0.05; Figure 6.3).

Postprandial coagulation *in vivo* and *in vitro*

There were no significant differences between the meal and fasting day for both F_{1+2} and TAT concentrations (Figure 6.4). Furthermore, Figure 6.5 shows the results of the fibrin generation test on MP-containing plasma performed at baseline and at time point t=6 hours (i.e. 6 hours after breakfast and 2 hours after lunch; these time intervals were chosen since

Table 6.2 Phospholipid composition of MP during postprandial or fasting state

		Time points (hours)				
Phospholipid	Meals	0	2	4	6	8
L-PC	+	1.3±0.8	0.9±0.8	1.0±0.8	1.3±0.8	1.3±0.8
	-	1.2±1.0	1.2±1.2	1.2±1.2	1.1±1.3	1.6±0.9
SM*	+	27±9.4	**21±3.6#**	23±5.5	22±4.5	22±4.7
	-	24±5.8	23±6.3	25±5.0	25±4.7	25±5.5
PC*	+	46±6.8	47±7.8	46±8.5	**49±10#**	48±13
	-	42±12	43±11	42±11	43±9.2	41±11
PS	+	8.1±6.6	12±7.5	9.2±7.3	8.2±7.2	9.1±6.7
	-	13±12	13±11	11±7.5	11±8.4	12±12
PI	+	6.1±3.8	6.4±3.6	**6.9±3.6#**	6.2±4.1	6.6±4.9
	-	5.7±4.0	6.9±3.8	8.4±7.3	6.6±3.3	6.7±3.1
PE	+	11±5.1	13±2.8	14±4.5	13±5.4	13±5.6
	-	13±6.5	12±5.0	13±8.3	14±5.0	13±3.9

Data expressed as % of total phospholipid (mol/mol) ±SE. *P<0.05 (area under the curve), #P<0.05 (individual time points). L-PC indicates lyso-phosphatidylcholine; SM, sphingomyelin; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

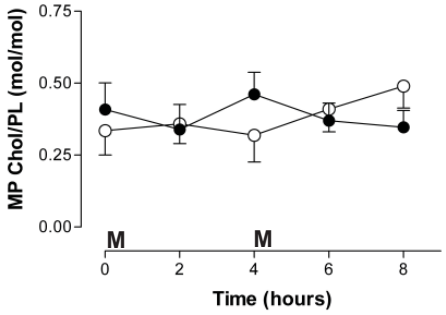

Figure 6.3 The cholesterol : phospholipid (PL) ratio of MP during the meal (solid circles) and fasting (open circles) days in healthy males. Meal intake is indicated by M. Data are mean±SE.

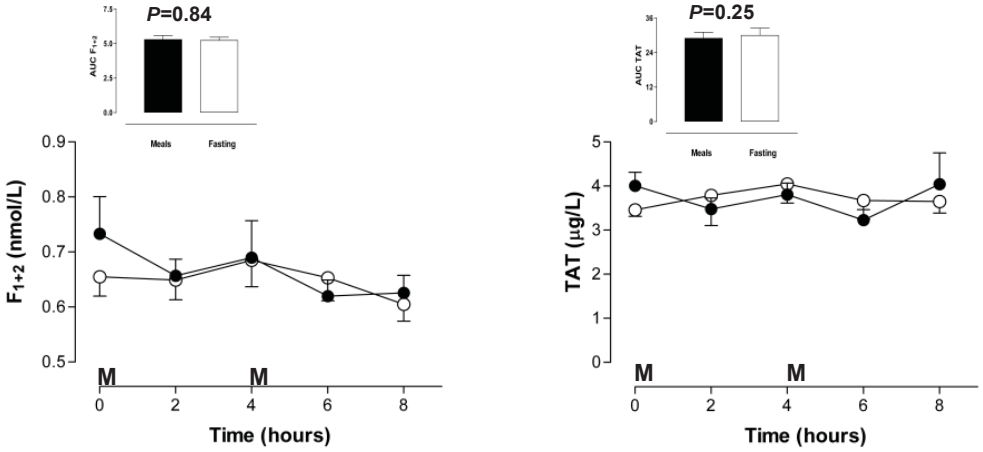

Figure 6.4 Plasma concentrations of F_{1+2} and TAT during the meal (solid circles) and fasting days (open circles) in healthy males. Meal intake is indicated by M. Bars (black, meals; white, fasting) in the insets represent respective AUC values. The P value is given for AUC difference. Data are mean±SE.

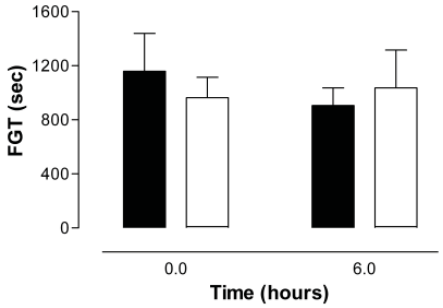

Figure 6.5 Fibrin generation tests (FGT) at baseline (t=0 hours) and at t=6 hours during the meal (black bars) and fasting (white bars) days. Data are mean±SE.

the metabolic changes were maximally different from baseline). There were no significant differences between the two days in the ability of MP to trigger fibrin formation in plasma in the absence or presence of anti-human factor VII, factor XI or factor XII.

DISCUSSION

The present study demonstrates that not only the numbers and cellular origin but also the phospholipid composition of circulating cell-derived MP changes after consumption of two consecutive high-fat mixed meals in healthy males. These changes, however, do not lead to enhanced coagulation activation *in vitro* and *in vivo*, suggesting that these relatively minor alterations in circulating MP are insufficient to cause a procoagulant state and that, under these physiological conditions, MP do not affect coagulation.

Surprisingly, in the postprandial state, the numbers of erythrocyte-derived MP markedly increased and these numbers were strongly associated with insulin concentrations. Since insulin induces a Ca^{2+}-dependent hyperpolarization of erythrocyte membranes, we hypothesize that this may be an inducer for the shedding of MP which is known to be highly calcium dependent.[23-25] Previously, we demonstrated that erythrocyte-derived MP are associated with factor XI-dependent coagulation in sickle cell disease, but we could not confirm this association in the present study. A possible explanation is that the changes in the phospholipid composition, especially the presence of oxidized and nonoxidized phospholipids, may differentially affect coagulation.[26]

In healthy individuals, cell-derived MP originate predominantly from platelets and contain mostly phosphatidylcholine and sphingomyelin, with smaller amounts of phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol, and minute quantities of the lysophospholipids lyso-phosphatidylcholine, lyso-phosphatidylethanolamine and lyso-phosphatidylserine. We showed that following 2 consecutive meals the relative amount of phosphatidylcholine increases, whereas concurrently sphingomyelin decreases in MP, compared to a fasting day. Furthermore, following a meal, the relative amount of phosphatidylinositol in MP also increases. These findings confirm earlier studies showing that changes in the phospholipid composition of MP vary between cell types and depend on the activation status of the parental cell,[21,27,28] e.g. platelet-derived MP originating from collagen-activated platelets contained less phosphatidylcholine and more sphingomyelin, phosphatidylethanolamine and phosphatidylserine than non-stimulated platelets.[27] Furthermore, the postprandial increase of phosphatidylinositol in MP following a high fat meal is in line with a previous finding showing that the amount of this phospholipid is increased in membranes from erythrocytes of diabetic patients.[29]

One of the important findings of the present study is that the total amount of phosphatidylserine present in MP fractions remained unaffected by fasting or meals in healthy subjects. Since phosphatidylserine exposed by MP or cells is believed to support the binding of (activated) coagulation factors and thus the formation of tenase and prothrombinase complexes, our present results may explain why we found neither differences in coagulation activation *in vivo* nor as coagulation activation potency *in vitro*.

In the present study, the phospholipid composition of *in vivo* MP was corrected for the plasma background. We found this correction to be essential, since both MP and MP-depleted plasma contained substantial quantities of phosphatidylcholine and sphingomyelin.[30] In contrast, the quantities of phosphatidylserine, phosphatidylinositol and phosphatidylethanolamine were below the detection limit of our assay in MP-depleted plasma, whereas phosphatidylserine, phosphatidylinositol as well as phosphatidylethanolamine were clearly detectable in the corresponding fractions of MP. This finding implicates that determination of the concentrations of phosphatidylserine-exposing microparticles or their coagulant properties by capturing the MP from plasma by using ELISA plates coated with annexin V may be a suitable tool for MP detection and possibly quantification *ex vivo*.

The observed increase in the cholesterol : phospholipid ratio of circulating MP during a prolonged episode of fasting and its association with plasma NEFA concentrations may be of interest. During the fasting day, the concentrations of NEFA in our healthy population increased almost 3-fold due to unsuppressed lipolysis, reaching levels that are found in fasting patients with type 2 diabetes.[31] Previously, it was shown that the amount of cholesterol in membranes of erythrocytes and leukocytes is associated with plasma fatty acids in patients with type 2 diabetes.[32] Furthermore, we demonstrated previously that MP released upon platelet activation have an increased cholesterol content.[21] It has been shown in several studies that growing cells in media with an altered balance of specific lipids, particularly oligo-unsaturated fatty acids and sterols such as cholesterol, affect the lipid composition of the plasma membrane.[33,34] Since cholesterol plays several structural and metabolic roles, particularly in membranes, cells may shed MP to cope with acute lipid changes in plasma to retain their membrane qualities.

In conclusion, the numbers, cellular origin and phospholipid composition of cell-derived MP change upon consumption of 2 consecutive high-fat mixed meals in healthy males. However, these changes do not seem to affect the ability of circulating MP to activate coagulation, both *in vivo* and *in vitro*. We therefore hypothesize that the observed differences in (phospho-)lipid composition of cell-derived MP on fasting and meal days reflect the ability to maintain membrane homeostasis, rather than to modify coagulation.

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8

Elevated endothelial microparticles following consecutive meals are associated with vascular endothelial dysfunction in type 2 diabetes

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INTRODUCTION

Type 2 diabetes (T2DM) is associated with prolonged and exaggerated postprandial hyperglycemia and hypertriglyceridemia, endothelial dysfunction and cardiovascular disease (CVD).[1-7] Endothelial dysfunction may link postprandial dysmetabolism to CVD.[5-7] Since the postprandial state unveils the full scope of metabolic abnormalities in T2DM, previous studies in fasting subjects may have underestimated the true risk.[1] Currently, endothelial functions can only be estimated from indirect measurements, such as flow-mediated dilation (FMD).[6,8]

Cell-derived microparticles (MP) are released by cells in response to stress. Increased numbers of MP of various cellular origin circulate in patients at risk of CVD.[9,10] Recently, VE-Cadherin (CD144) positive MP were demonstrated in T2DM patients with coronary artery disease and patients with end-stage renal disease.[11,12] Since VE-Cadherin is exclusively expressed by endothelial cells, CD144-positive MP may be regarded as endothelium-derived MP (EMP), directly reflecting endothelial damage. However, it is unknown whether circulating EMP are cause or consequence of CVD and whether their occurrence associates with CVD per se or rather with diabetes-related metabolic abnormalities.

We hypothesize that exposure of patients with uncomplicated T2DM to 3 consecutive high-fat mixed meals, given during a 24-h period, will disclose the full scope of their compromised metabolism and subsequent endothelial dysfunction, measured as FMD and circulating EMP.

RESEARCH DESIGN AND METHODS

After obtaining informed consent, 27 non-smoking Caucasian males (n=15 with uncomplicated T2DM, n=12 healthy age-matched volunteers) were studied during a 24-h period. No drug use other than sulfonylureas and/or metformin was allowed. After an overnight fast, subjects received 3 consecutive, isocaloric (900 kcal), mixed meals (75g carbohydrates, 50g fat (60% saturated), 35g protein), at time points t=0 (breakfast), 4 hours (lunch), and 8 hours (diner). The study was approved by the local ethics committee and conformed to the principles of the Declaration of Helsinki.

Venous blood samples were collected before and at fixed intervals following the first meal as described previously.[13] To avoid artifacts, no in-dwelling canulae were used and for each blood collection a new collecting system (Microflex, size 1.0mm - 19G, Vygon, France) was employed. Plasma aliquots (250 μL) were obtained after centrifugation (1550*g*, 20 minutes, 20°C) and snapfrozen in liquid nitrogen and stored at -80°C until assay. Glucose, HbA1c, insulin and lipids were measured as previously described.[13]

MP were isolated and analyzed as described using a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA).[9,13] MP were identified on forward scatter (FSC), sideward scatter (SSC), binding of annexin-V and a monoclonal antibody directed against a cell type-specific antigen, and quantified as described.[9,13] Samples were analyzed by a blinded technician.

Before each blood collection, FMD was measured at the right brachial artery by a single observer (CV<2%) using ultrasound (Wall-track System, PieMedical, Maastricht, The Netherlands).[8,13]

To estimate the overall changes during 24-h, the area under curve (AUC) of metabolic parameters, FMD and MP, plotted against time was calculated. Data were analyzed by repeated-measures ANOVA, with time of measurement as the within factor and group as the grouping factor. Non-normally distributed data were log transformed before analysis, otherwise, non-parametrical tests were performed. Post hoc tests were only performed when ANOVA revealed overall significant differences. Correlations were performed using the Spearman's test. A $P<0.05$ was considered statistically significant.

RESULTS

At baseline, T2DM patients (mean age 55±2 yrs) had higher BMI, blood pressure, HbA1c (7.1±1.1%), plasma glucose, triglycerides and insulin levels, but lower HDL-C than controls (data not shown).

Plasma glucose, triglyceride and insulin concentrations rose postprandially in patients, relative to baseline and healthy controls (Figure 8.1A; all $P<0.05$). AUC glucose, triglycerides and insulin concentrations were significantly higher in patients vs. controls (all $P<0.02$).

At baseline, FMD was decreased in patients vs. controls (5.5% vs. 8.9%; $P<0.01$), and deteriorated postprandially in both groups (Figure 8.1B). FMD during 24-h was reduced in patients vs. controls ($P<0.01$) and was inversely associated with AUC glucose, triglycerides and insulin (r=-0.77, r=-0.52 and r=-0.59, all $P<0.01$, respectively).

Total MP at baseline and AUC of total MP were similar in patients and controls ($3.2x10^9$/L vs. $3.5x10^9$/L, P=0.32). In patients, the CD144-EMP fraction at baseline was higher than in controls (1.9%±2.0 vs. 0.8%±1.1, $P<0.05$), and rose significantly to 2.2±4.6% (vs. controls: 0.6±0.9%, $P<0.05$) at t=12h, i.e. after consumption of all 3 meals (Figure 8.1C).

AUC CD144-EMP was positively correlated with AUC glucose, triglycerides and insulin (r=0.51, r=0.38 and r=0.57, all P<0.05, respectively) and negatively associated with FMD during 24-h (r=-0.60, p<0.01; Figure 8.1D).

When 5 subjects per group were additionally studied during a 10-h fast, no increase of CD144-EMP was found (data not shown).

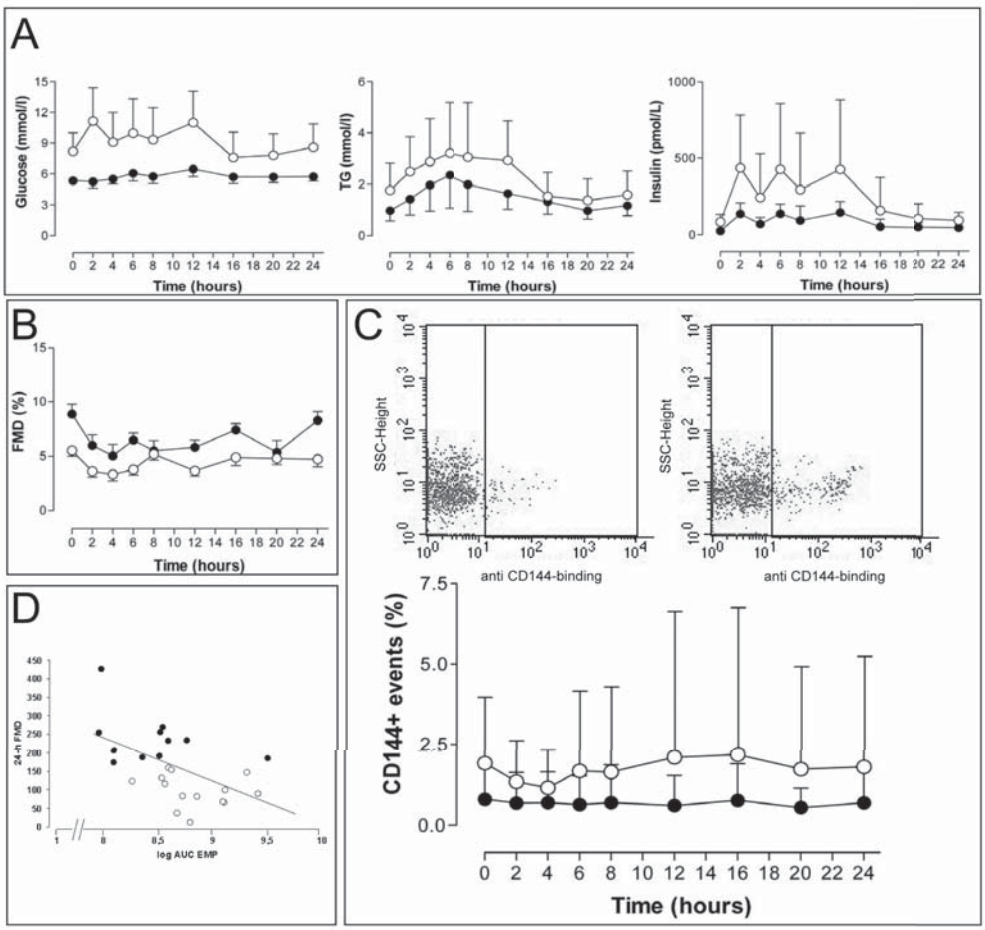

Figure 8.1 **A:** The 24-h course of plasma glucose, triglyceride, and insulin concentrations in T2DM (open circles) and healthy subjects (solid circles). **B:** Changes in flow-mediated dilation (FMD) in T2DM (open circles) and healthy subjects (solid circles) during the 24-hour study day. **C:** Representative FACS dot plots of endothelium-derived MP (EMP) of a T2DM patient before (left) and after (right panel) 3 high-fat mixed meals and the 24-h course of EMP in T2DM (open circles) and healthy subjects (solid circles). Data in A, B and C are mean±SD. **D:** Scatter plot representing the relationship between FMD during 24-h and EMP in T2DM (open circles) and healthy subjects (solid circles).

CONCLUSIONS

CD144-MP circulate in patients with uncomplicated T2DM and associate with postprandial metabolic derangements and impaired FMD. We mimicked a real-life situation by giving T2DM patients 3 consecutive meals to unveil the true burden of meal-induced metabolic disturbances during a 24-h period.[1] These derangements coincided with the highest levels of CD144-MP, which are derived from endothelial cells only. Other investigators used CD31 (i.e. PECAM-1) to identify EMP.[14] However, CD31 is not only present on endothelial cells, but also on platelets and platelet-derived MP, the latter being the most abundant MP in the circulation.[10,15] Therefore, previously reported EMP numbers may be an overestimation of the actual EMP.

Under physiological conditions, the ability of cells to release MP may reflect their capacity to cope with stress.[9,10] By shedding caspase 3-containing MP, cells may prevent apoptosis and maintain homeostasis.[9] In disease, however, the ability to handle cell stress and to release MP may be altered. This may explain the difference between our present and earlier findings, showing that in well-trained young healthy males the total numbers of circulating MP rose postprandially, whereas in T2DM patients no diurnal changes in total MP were observed and only a trend towards total MP elevation was observed in controls (data not shown). Thus, in T2DM, the ability of (endothelial) cells to respond to excessive metabolic stress may be impaired.

To summarize, in uncomplicated T2DM, consumption of high-fat meals results in dysmetabolic changes and subsequent endothelial stress and injury, thereby contributing to atherogenesis and CVD risk. The possible value of CD144-MP as a marker to quantify endothelial dysfunction and/or injury needs further exploration.

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9

Cell-derived microparticles in the pathogenesis of cardiovascular disease: friend or foe?

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ABSTRACT

Microparticles are ascribed important roles in coagulation, inflammation and endothelial function. Inasmuch as these processes are mandatory to safeguard the integrity of the organism, their derangements contribute to the development of atherosclerosis and cardiovascular disease. More recently, the presumed solely harmful role of microparticles has been challenged, as microparticles may also be involved in maintenance and preservation of cellular homeostasis, and in promoting defense mechanisms. Here, we summarize recent studies revealing these two faces of microparticles in cardiovascular disease.

INTRODUCTION

Cardiovascular disease (CVD) remains the leading cause of death in developed countries despite advances in treatment.[1] Although assessment and treatment of traditional cardiovascular risk factors, including hypertension, diabetes and smoking, play a central role in primary and secondary disease prevention, even adequate treatment of these risk factors does not completely reduce the risk of CVD. Therefore, a quest for novel biomarkers or mediators is ongoing, which may help us to gain more insight into the mechanisms leading to the development of atherosclerosis, the underlying cause of CVD.[2] Cumulating evidence suggests that microparticles, i.e. vesicles budded from the outer membrane of cells upon their activation or as part of the apoptosis of the cell, and other microvesicles, may provide such a mediator. Since their identification in the late 60s of the previous century, the scientific and clinical interest in cell-derived microparticles has increased substantially. This is not surprising, because microparticles affect not only coagulation and inflammation, but also endothelial function, cellular survival and intercellular communication.[3-5]

The majority of microparticles in blood is derived from platelets, whereas microparticles from erythrocytes, granulocytes, monocytes, lymphocytes and endothelial cells are also present, albeit at much lower numbers. There are substantial differences between the fractions of microparticles or subpopulations in blood of healthy subjects and those present in patients suffering from diseases with increased thromboembolic risk or vascular damage, such as atherosclerotic vascular disease, sepsis, diabetes mellitus, chronic severe hypertension and preeclampsia.[3-5] Accordingly, in patients with acute myocardial infarction elevated numbers of microparticles are present compared to healthy controls.[3,6,7] Moreover, subtypes of microparticles differ between patients with stable angina and acute coronary syndromes or myocardial infarction.[7,8] Earlier it was demonstrated that microparticles from patients with myocardial infarction cause endothelial dysfunction and might contribute to the general vasomotor dysfunction observed after myocardial infarction.[9]

The clinical relevance of the presence of microparticles in blood of healthy subjects is as yet unclear, but can be regarded as a reflection of the dynamics of their production by resting, activated and apoptotic cells, and their clearance. In vascular disease states it remains to be elucidated whether microparticles are a cause or a consequence of the condition, since disease-related factors such as infectious agents, cytokines and metabolic disturbances are all known to affect the release of microparticles.[3-5] Still, it is likely that microparticles contribute to the severity of disease, as microparticles can disseminate procoagulant and proinflammatory activities. Therefore, microparticles may be viewed as part of a cascade of reactions in response to various stimuli. The stimulus that leads to their generation determines their numbers, size, biochemical composition and functional characteristics.

Microparticles are likely to be important and essential mediators of physiological and pathological conditions, and in this review we will focus on the clinical relevance of microparticles in CVD.

MICROPARTICLES: FRIEND OR FOE?

All body fluids, ranging from blood and urine to lacrima and mother milk, and human atherosclerotic plaques contain cell-derived vesicles. Microparticles as outer membrane-derived vesicles are one type of vesicles, as are exosomes, which are extruded from intracellular multivesicular bodies once these fuse with the outer membrane. Different types of vesicles originating from a variety of cells are concurrently present in our body fluids under physiological and pathological conditions. Most clinical studies thus far have focused on the presence of microparticles in blood, their cellular origin, composition and function in disease.[3-5]

Microparticles may have not only deleterious effects by promoting coagulation and inflammation, or by modifying endothelial function, which all contribute to development of CVD, but may also have beneficial effects. First, recent studies have shown that microparticles are efficient vectors that exchange biological information between cells (intercellular communication).[3-5] Second, the release of microparticles protects cells against the consequences of external stimuli or stress. Endothelial cells escape from complement-induced lysis by releasing microparticles carrying the lytic complement C5b-9 complex.[10] Similarly, the release of microparticles protects cells against an overshoot in (internal) cellular reactions triggered by external stressors. With regard to the latter, microparticles play a role in "cellular waste management", as they contain increased (compared to the parent cell) concentrations of chemotherapeutics, oxidized phospholipids or caspase 3.[3-5] Microparticles of viable, non-apoptotic cells, including endothelial cells, contain caspase 3 *in vitro* and *in vivo*.[11-13] Endothelial cells accumulate caspase 3 and undergo apoptosis when the release of (caspase 3-containing) microparticles is inhibited,[13] indicating that the release of microparticles is part of a protective mechanism to prevent the intracellular accumulation of caspase 3 at dangerously high levels. Possibly, caspase 3 facilitates its own removal from cells by cleaving several kinases, including Rho-associated kinase I (ROCK-I) and p21-activated kinase (PAK), which become constitutively active upon cleavage and promote membrane blebbing. Upon incubation of human endothelial cells with simvastatin at clinically relevant concentrations, the cells remain viable and seemingly unchanged, but a marked increase in caspase 3-containing microparticles is observed, suggesting that an increased release of caspase 3-containing microparticles is needed to help the cells to remain

healthy and viable.[14] It should be mentioned that the effects of statins on the release of microparticles may depend on the cell types studied, the experimental conditions including the concentration and type of statin used, or the combined application of a statin with a pro-inflammatory or pro-apoptotic inducer, but it is beyond the scope of this brief review to elaborate on this topic. In line with our results are the findings that in patients with subclinical or less occlusive atherosclerosis, higher numbers of endothelial microparticles are present compared to patients with established or symptomatic atherosclerosis,[8,15] suggesting that the ability of the endothelium to release microparticles depends on its integrity and viability. In other words, if the ability of the endothelium to release microparticles becomes impaired or inhibited, the integrity and viability of the cells may deteriorate.

Microparticles and coagulation

The most described and reviewed characteristic of circulating and atherosclerotic plaque-derived cell-derived microparticles is their procoagulant phenotype. By exposing phosphatidylserine and sometimes tissue factor (TF), microparticles can initiate and propagate coagulation.[3-5] However, TF seems often to be exposed by circulating microparticles in a non-coagulant form, as shown in patients with type 2 diabetes.[16] This inactive or encrypted TF may become pro-coagulant after the capturing of the TF-bearing microparticles in the developing thrombus.[17] This is supported by the recent finding that those patients with acute ST-segment elevation myocardial infarction, who had the highest plasma level of TF-exposing microparticles, were characterized not only by a pro-coagulant phenotype as reflected by increased plasma levels of thrombin-antithrombin complexes, but also by an increased risk of fibrinolysis failure.[18] Furthermore, microparticles accumulate in human atherosclerotic plaques, and these microparticles have an increased ability to initiate tissue factor-dependent coagulation compared to the plasma microparticles.[19]

The capturing of TF-bearing microparticles in the developing thrombus, that finally results in arterial occlusion, is thought to be mediated by P-selectin and / or CD36 exposed on activated platelets.[17,20] Important factors in de-encryption of the TF procoagulant activity are protein disulfide isomerases (PDI), believed to be released from platelets and endothelial cells. In addition, platelet microparticles can expose PDI.[21] Very recently, it was demonstrated that fibrin generation *in vivo* required PDI released from endothelial cells, whereas platelet-derived PDI contributed only to the total amount of thrombus-associated PDI but was not required.[22]

The relationship between microparticles and coagulation as well as other processes is very complex. For instance, under normal conditions a continuous low grade generation

of thrombin is essential for activation of protein C, which in turn efficiently inhibits additional thrombin generation by inactivating coagulation factors Va and VIIIa. Previously, we reported that plasma samples from healthy human individuals containing low levels of microparticles have relatively high concentrations of thrombin-antithrombin and prothrombin fragment 1+2, suggesting that in these subjects insufficient activation of protein C may occur.[23-25] Under clinical conditions, where elevated levels of microparticles can be present and where microparticles may expose coagulant TF, this inverse correlation is no longer present and then microparticles are more directly associated with *in vivo* coagulation activation.

Microparticles are now thought to contribute to acute thrombus formation via various mechanisms, but also to contribute to anti-coagulant responses to modulate an adequate and physiologic coagulation response. Additional research, however, is essential to better understand their contribution to coagulation in clinical conditions, including CVD.

Microparticles and inflammation

Similar to coagulation, inflammatory processes contribute to the pathogenesis of atherothrombotic vascular disease.[26] Patients with elevated plasma levels of inflammation markers, including C-reactive protein (CRP), are at a high-risk to develop CVD, including those with diabetes mellitus, the metabolic syndrome, as well as those with chronic systemic inflammatory or autoimmune disease such as patients with rheumatoid arthritis (RA).[26,27] Microparticles may contribute to inflammatory responses by various mechanisms.[3-5] For example, microparticles from leukocytes stimulate the expression of proinflammatory genes in endothelial cells, leading to the production of cytokines and leukocyte-endothelial cell adhesion molecules *in vitro*.[28] Furthermore, microparticles in plasma samples from high-risk patients expose complement components C1q, C3 and C4, as well as several complement activator molecules, including CRP in patients with RA and IgG but not CRP in patients with myocardial infarction.[29,30]

But again, microparticles may also have a beneficial function in the inflammatory response. Microparticles from polymorphonuclear leukocytes contain the functionally active anti-inflammatory protein annexin 1 (AnxA1), and AnxA1-containing microparticles inhibit the interaction between leukocytes and endothelial cells *in vitro* as well as in an animal model *in vivo*.[31] So, in health, microparticles can affect both pro- and anti-inflammatory processes, thus assuring an appropriate inflammatory response. In diseases such as RA or atherosclerosis, their increased and prolonged presence, altered properties and activities may become harmful and contribute to overall vascular deterioration.

Microparticles and endothelial functions

Under normal conditions, the multiple functional characteristics of the endothelium, that in addition to anti-inflammation and anti-coagulation include the regulation of the vascular tone, vascular wall permeability, and cell growth collectively protect the vascular system. An altered function of endothelial nitric oxide (NO) synthase (eNOS) and/or decreased bioavailability of NO are fundamental abnormalities that can lead to the pathophysiological manifestations of endothelial dysfunction.[32] *In vitro*, microparticles from various cellular and/or disease origin induce endothelial dysfunction, especially by altering the balance between NO and reactive oxygen species (ROS) production and release.[33-35] For example, microparticles from T-lymphocytes decrease the NO production and increase oxidative stress in endothelial cells.[35] These effects are associated with a reduction of eNOS activity, which depends on phosphatidylinositol-3-kinase (PI3K), ERK½, and NF-κB pathways. The increase of ROS production that is down-regulated by the PI3K pathway involves xanthine oxidase and NF-κB pathways. Also, exposure to microparticles results in increased caveolin-1 expression and decreased phosphorylation, respectively, these effects being independent of the PI3K and ERK½ cascade.[35] *In vivo*, NO-associated vasodilatation following shear stress can be measured by ultrasound of the brachial artery (flow-mediated dilatation (FMD)), and is associated with the viability of the endothelium. Of interest, an impaired FMD has been associated with the presence of endothelial microparticles in various clinical conditions.[36,37]

In contrast to the harmful role of microparticles, they may actually improve endothelial dysfunction. T-cell microparticles carrying the morphogen sonic hedgehog (Shh) induced NO production directly by the Shh pathways (involving PI3-kinase and Akt).[38] Using a model of ischemia/reperfusion in mice, these Shh-presenting microparticles enhanced NO-mediated relaxation of mouse coronary arteries *in vivo* in response to acetylcholine, which was accompanied by an increase of NO production in tissues and blood even after ischemia/reperfusion. Moreover, microparticles, especially those of non-platelet origin, released during sepsis, the clinical condition that ultimately challenges the endothelium, were protective against vascular hypo-reactivity which accounts for hypotension in patients with septic shock.[39] In agreement with these results, Soriano and coworkers have shown that elevated levels of endothelial and platelet microparticles predict a more favorable outcome in severe sepsis in terms of mortality rate and organ dysfunction.[40] Evidently, microparticles are able to restore endothelial injury through their dual ability to increase NO and reduce ROS. In summary, microparticles can have both detrimental and beneficial effects on endothelial functions, especially by altering the balance between NO and ROS production and release. It seems that these effects are depending on the specific stimulus underlying the release of microparticles by their parent cells.

Finally, it should be mentioned that circulating as well as atherosclerotic plaque-derived microparticles can affect angiogenesis. One characteristic feature of vulnerable atherosclerotic plaques is an increased number of vasa vasorum. Whereas in initial studies microparticles from platelets and endothelial cells were shown to promote and inhibit angiogenesis *in vitro*, respectively, more recently Leroyer and coworkers showed that microparticles isolated from human atherosclerotic plaques not only promote endothelial cell proliferation *in vitro* but also stimulate angiogenesis *in vivo*. Thus, microparticles may contribute to neovascularization of atherosclerotic plaques, thereby affecting the vulnerability of rupture.[41-43]

Microparticles and cardiovascular disease

In the light of the above-described pro-coagulant and pro-inflammatory properties of microparticles, together with the association between elevated numbers of microparticles and clinical CVD, the prevailing view is that circulating microparticles are harmful, contributing to CVD and risk thereof. However, as outlined above, besides their potentially harmful effects, cell-derived microparticles may also be beneficial and as such protect against cellular and vascular damage. This is summarized in Figure 9.1. Therefore, it is not surprising that both elevated as well as lower levels of circulating microparticles have been associated with (risk factors of) CVD. For example, cigarette smoking is a well established risk factor for CVD and has been reported to lead to haemostatic, platelet and endothelial abnormalities.[44] Whereas the numbers of platelet microparticles tended to be lower in young males compared to non-smoking controls, levels of circulating endothelial microparticles were elevated upon secondhand smoke exposure, and exposure of human monocytes to smoke increased the release of (TF-exposing) microparticles.[45-47] It is clear that smoking affects the release of microparticles, but this effect is likely to be cell-type, concentration and duration dependent.

Interestingly, elevated platelet microparticles were described in patients with both type 1 and 2 diabetes mellitus, those with hyperlipidemia, obesity / the metabolic syndrome and hypertension.[3,5] Thus, in plasma samples from patients with chronic severe hypertension compared to patients with mild hypertension and controls, higher numbers of microparticles exposing PECAM-1 (CD31) but not glycoprotein Ib (CD42), i.e. microparticles presumably of endothelial and/or platelet origin, were found.[48,49] In these patients, elevated numbers of microparticles are likely to reflect the cellular stress of endothelial cells and platelets. In addition, microparticles from plasma of hypertensive patients with albuminuria attenuated proliferation and migration of endothelial progenitor cells, and increased endothelial hydrogen peroxide production, cellular senescence and apoptosis compared

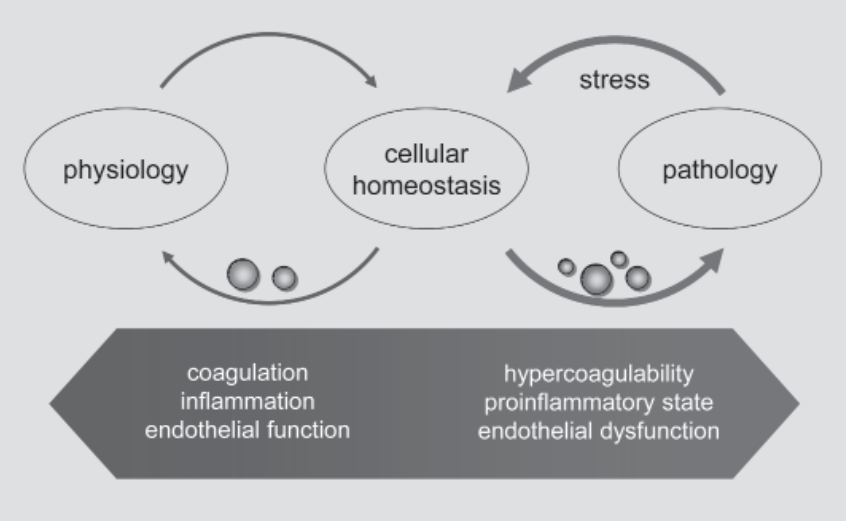

Figure 9.1 Proposed model describing the role of microparticles in cardiovascular health and disease. Cells release microparticles (left, green) to ensure homeostasis. These microparticles contribute to the regulation of physiological processes including (anti)coagulation, inflammation and regulation of endothelial functions. Under abnormal (stress) conditions, cells release microparticles that differ in numbers, composition and function (right, red), thereby contributing to a procoagulant and proinflammatory phenotype, endothelial dysfunction, as part of the development of cardiovascular disease.

to microparticles from hypertensive patients with normoalbuminuria.[50] This suggests that the release of microparticles contributes to the progression of endothelial damage and this could then result in a vicious circle.

High numbers of circulating microparticles of different cellular origin have been associated with subclinical atherosclerosis, measured by carotid intima-media thickness (cIMT) and coronary artery calcifications, quantified by computer tomography, as described by several authors.[8,15,51-53] Recently, it was proposed that circulating microparticles are the major factor associated with carotid artery remodeling, with high numbers of circulating microparticles preventing compensatory remodeling in vessels with increased cIMT.[53] Moreover, T-cadherin-exposing endothelial microparticles were elevated in patients with subclinical atherosclerosis, not only compared to healthy subjects but also to patients with established coronary artery disease, suggesting that the shedding of T-cadherin-positive microparticles represents a protective mechanism that can shift the balance in cellular stress response to the pro-survival signaling branches, which is only later followed by deleterious and apoptotic

phases.[15] This indicates that the release of microparticles attenuates compensatory outward artery remodeling in patients with (subclinical) atherosclerosis. However, longitudinal rather than the presently available cross-sectional studies are required to definitively demonstrate the causative role of microparticles in arterial remodeling.

An important clinical question is whether microparticles are markers of CVD risk and whether they can be used as independent predictors of CVD outcome in relevant populations. Recently, the associations of the Framingham Risk score and numbers of platelet-, leukocyte-, endothelial progenitor-cell- and endothelium-derived microparticles where reported.[52,54-56] From these four studies, only one included a prospective investigation, in which 488 patients with various CVD risk factors were followed for a mean period of 36 months.[56] The addition of endothelial (CD144+)-microparticles to the Framingham risk model improved not only the classification of risk, but also appeared as a significant and independent predictor of future CVD events in a high-risk population.[56] These results are promising and imply that more prospective studies are needed to further detail the prognostic value of cell-derived microparticles in individuals at high risk for CVD. The lack of large-scaled studies in which microparticles are measured routinely may be partly due to the fact that to date the measurement of microparticles is still complex and elaborate. The detection and characterization of (individual) microparticles and other vesicles remains difficult due to their small size and heterogeneity, which has lead to confusing and sometimes conflicting results between laboratories.[57] In addition, the detection of rare microparticles, e.g. endothelial microparticles, remains a real challenge. Whereas some laboratories have used antibodies against specific endothelial proteins, other investigators have used non-endothelial specific antibodies or combinations thereof.[57,58]

CONCLUSION

Since their presence and role in various diseases has been recognized, the interest in cell-derived microparticles, also as markers or as mediators in the development of CVD, has grown substantially. Over the past years, the role of microparticles in the development of CVD has changed from "platelet-dust" or artifacts to novel and possibly essential elements in cellular homeostasis and communication. Furthermore, the release of microparticles contributes to the activation of (intentionally protective) protein cascades such as coagulation, inflammation, and also endothelial dysfunction (Figure 9.1), which all facilitate atherogenesis. Therefore, microparticles may not only reflect the presence of CVD, but also play a causative role in the development of CVD.

On the other hand, the release of microparticles can protect cells from dangerous or redundant products, compounds or cellular waste that may accumulate in response to cell stress induced by e.g. CVD risk factors. Thus, microparticles can contribute to cellular homeostasis. Although the release of microparticles may be beneficial to the individual cell releasing these microparticles, this release can also have paracrine and even systemic effects, affecting the surrounding cells. Whether cell-derived microparticles are a cause and / or a consequence of CVD remains to be established. Large-scaled prospective studies rather than the presently available cross-sectional studies, are mandatory to more definitively demonstrate the interrelation of changes in occurrence, phenotype and function of microparticles and the incidence and progression of CVD in various populations.

FUTURE DIRECTIONS

Future research should focus on further characterisation of cell-derived microparticles as opposed to exosomes and possibly other cell-derived vesicles in various diseases. Moreover, multidisciplinary research should focus on further refinement and validation of detection methods of microparticles. These methodological investigations may contribute to more uniformity and validation of microparticle quantification, detection, identification of their parent cell and biochemical and functional characterisation. With a multidisciplinary approach and the proper verification studies in relevant patient populations, microparticles may prove out to be true biomarkers of disease state and progression.

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General discussion and future perspectives

The prevalence of obesity is reaching to pandemic proportions, mainly because of ageing, and global adopting of the so-called Western lifestyle, i.e. a high intake of energy dense food and a low physical activity pattern.[1] These life-style changes lead to one of the key abnormalities underlying type 2 diabetes mellitus (T2DM), i.e. insulin resistance, which occurs often in the presence of central obesity, and the sequelae, i.e. hyperinsulinemia, hypertension and dyslipidemia. Collectively, these often concurring cardiometabolic derangements constitute the so-called metabolic syndrome (MetS).[2] Separately, the features of the MetS are regarded as risk factors for the development of cardiovascular disease (CVD),[3] and the CVD risk increases proportionally to the number of features present.[4,5] In susceptible individuals with (genetically) compromised beta-cell function and/or beta-cell functional mass, the constant demand on the beta-cells will ultimately lead to beta-cell failure with ensuing hyperglycemia and T2DM. In insulin resistant individuals with T2DM, the metabolic abnormalities become even more pronounced upon challenge, such as in response to a meal, resulting in prolonged hyperglycemia, hypertriglyceridemia, elevated fatty acids and hyperinsulinemia. In these individuals, due to these prolonged and exaggerated metabolic derangements, all organ systems, including the heart and the vascular endothelium, are exposed to a pro-atherogenic, pro-inflammatory and pro-thrombotic milieu for almost 24 hours a day. As the abnormal metabolic responses usually concur, it makes it more difficult to tease out their relative contribution to cardiovascular damage and to design studies focusing on the effects of a single factor. Furthermore, seemingly distinct mechanisms of action, when concurring, interact and produce synergistic increases in oxidative stress, protein kinase-C (PKC) activation and advanced glycation end-product receptor (RAGE) activation.[6,7] Collectively, these derangements result in systemic and vascular inflammation, impaired endothelial function and ultimately, in CVD.[8,9] Importantly, chronic inflammation and elevated oxidative stress are not only associated with the complications of obesity or diabetes, but have also been linked to insulin resistance *in vitro* and *in vivo*.[10-12]

In this thesis, it is attempted to obtain a more detailed understanding of the previously reported relationship between the postprandial state and cardiovascular damage in high risk individuals. Accordingly, underlying mechanisms, including oxidative stress, endothelial function and quantitative and qualitative changes in cell-derived microparticles (MP), linking postprandial dysmetabolic alterations to vascular abnormalities were explored. Also, to this end, we as much as possible wanted to mimic a real life situation and to tease out the additional contribution of hyperglycemia to the cellular and vascular changes, relative to the clustered risk factors common to the insulin resistant states, i.e. hyperinsulinemia, hyperlipidemia and hypertension. In order to accomplish all these goals, both the choice of

the population and the study design were instrumental. Thus, in these mechanistic studies, we chose to investigate males only, in order to have a more homogeneous population: women, relative to men, once they develop T2DM have a disproportional increase in the relative risk of macrovascular disease,[13,14] as well as different lipid profiles and importantly, care should be taken that all are postmenopausal to avoid further confounding of different hormonal states.[15-17] Thus, males with the MetS (in whom the MetS feature of dysglycemia was an exclusion criterion), males with the MetS and additional hyperglycemia, i.e. T2DM, and healthy subjects in the postprandial state were studied. The real-life situation was mimicked by a 10-h or 24-h study period during which the participants were given 2 (10-h period), or 3 (24-h period) consecutive standardized high-fat mixed meals, given as breakfast and lunch or as breakfast, lunch and dinner, respectively.

Major findings

This thesis in fact has two parts, i.e. the first part in which we detail quantitative and qualitative aspects of postprandial changes, particular with respect to the pro-atherogenic lipid profile, in men with MetS and in those with T2DM, versus controls. Furthermore, we also studied the *consequences* of these unfavorable postprandial responses on (markers of) oxidative stress and inflammation as well as vascular (functional) changes, but also on *causal* factors leading to these postprandial derangements, such hepatic steatosis (**Chapters 2-4**). In the second part, we focus on the role of MP, as potential mediators of the metabolic and vascular derangements, by detailing differences in their numbers, cellular origin, subpopulations and functional characteristics between those with MetS and T2DM and healthy individuals (**Chapters 5-8**).

PART I

Abdominal, particularly visceral obesity, is strongly associated with hepatic steatosis or non-alcoholic fatty liver disease (NAFLD), both via increased delivery of free fatty acids to the liver and through increases of hepatic lipogenesis associated with hyperglycemia and hyperinsulinemia.[18,19] In turn, the worsening insulin resistance associated with hepatic steatosis may lead to the development and/or exacerbation of the features of the MetS. The close associations among hepatic steatosis, obesity, and cardiometabolic risk factors have led to the suggestion that hepatic steatosis may be a novel component of the MetS,[18] whereas it may alternatively be regarded as key causal factor of the MetS. One mechanism that may partly explain the link between hepatic steatosis and CVD is the postprandial dysmetabolic state. Indeed, in males with T2DM and males with and without the MetS,

we demonstrated that the amount of liver fat is associated with pro-atherogenic changes within the postprandial lipid profile (**Chapter 2**). These results partly confirm and extend the observations by Matikainen et al., who found an elevated VLDL-TG output following a single meal in individuals with hepatic steatosis (with and without T2DM).[20] In addition to these findings, our results suggest an increased production or retainment of apoB-48 chylomicrons by the intestine in T2DM males following 3 consecutive meals.

Furthermore, we showed that hepatic steatosis was related to triglyceride enrichment of HDL particles in the postprandial state, resulting in changes of the physiochemical properties of HDL (**Chapter 4**).This postprandial triglyceride-enrichment of HDL particles altered the anti-oxidative capacity of this lipoprotein class, which was paralleled by endothelial dysfunction measured by ultrasound as flow mediated dilatation (FMD). Our findings based on a physiological stimuli, confirm but rather extend recent results by Patel et al. who demonstrated that infusion of an artificial fat emulsion (Intralipid) results in HDL-TG-enrichment with impaired endothelial function, as assessed by inhibition of (*in vitro*) endothelial cell adhesion molecule expression, in young healthy males.[21]

The pro-atherosclerotic functional changes in HDL and LDL particles during the postprandial state were studied in **Chapter 3**. We showed that prolonged hypertriglyceridemia, as observed in males with the MetS and patients with T2DM, following 3 consecutive meals, resulted in the formation of triglyceride-enriched HDL and LDL particles as well as concomitant increased postprandial susceptibility of LDL to oxidation. Furthermore, enlarged lipoprotein particles and impaired meal-related anti-oxidant capacity of HDL was specifically observed in T2DM. With these findings we confirm Diwadkar et al. demonstrating that postprandial LDL particles of diabetic subjects had a significantly shorter lag phase following one rather artificial meal, containing 85 g of fat,[22] and extend these findings by showing the association with changes in triglyceride content. The anti-inflammatory/oxidative properties of HDL in the postprandial state have been studied earlier in two relatively small studies with healthy subjects. Nicholls et al. showed that the anti-inflammatory potential of HDL was reduced after consumption of saturated fat and improved following the consumption of polyunsaturated fat.[23] As described previously, Patel and coworkers demonstrated an impaired anti-inflammatory capacity of HDL in response to 20% Intralipid.[21] We extend these findings by measuring endothelial function *in vivo*, using consecutive meals to mimic a more real-life situation and studying males with T2DM and males with MetS, compared to healthy males.

Taken together, our results show that the postprandial state is associated with changes in physiochemical properties of lipid particles, increased oxidative stress, and endothelial dysfunction, and that this is exaggerated and prolonged in dysmetabolic states like the MetS

and T2DM. In addition, increased liver fat accumulation contributes to these postprandial derangements. However, routine ultrasound examination of the abdomen for the screening of hepatic steatosis in combination with serologic tests for viral hepatitis infection may not be feasible in daily practice in most countries. Furthermore, in daily practice the availability of magnetic resonance spectroscopy (MRS) to measure liver fat content is scarce and expensive. In addition, performance of regular liver biopsies to survey liver fat content is not recommended because of its risks, discomfort and relative inaccuracy. Previously, we and others suggested alanine aminotransferase (ALT) as a marker of NAFLD. Accordingly, in the population-based Hoorn Study we showed that ALT levels in the high range of the normal distribution are associated with an increased 10-year risk of coronary heart disease in elderly individuals.[24,25] This association was independent of classical CVD risk factors and components of the Adult Treatment Panel III–defined MetS, indicating that ALT may be a useful marker in the assessment of CVD risk in patients who may have NAFLD. Although the evidence that lowering the amount of liver fat content is associated with decreased incidence of CVD is lacking, it is known that body weight reduction as a result of a negative energy balance due to dietary interventions and/or increased physical activity results in decreases in liver fat. Thus, any benefit obtained from body weight loss may be in part mediated by the metabolic improvements associated with concomitant reduction in liver fat. Still, in the absence of direct evidence from appropriate long-term intervention trials, it seems that early identification of patients with NAFLD and the associated cardiometabolic derangements may be important, not only in terms of risk for progression of severe steatosis into NASH, but also for adequate preventive and therapeutic measures aimed at reduction of CVD risk.

PART II

A more recent mechanism proposed to constitute a link between cardiometabolic risk factors and cardiovascular damage is the formation of pro-inflammatory and pro-coagulant MP. Elevated circulating numbers of these vesicles of various cellular origin and composition, have been described in many high-risk populations and associated with (markers of) cardiovascular damage and even cardiovascular events.[26-28] However, most human studies have focused on quantitative (circulating numbers) rather than qualitative aspects of MP, that were mostly of platelet origin and had been isolated from a single plasma sample obtained in the fasting state. At the time of the initiation of our studies, no data were available regarding 1) numbers and characteristics of MP in humans with uncomplicated T2DM versus MetS versus controls; 2) the impact of meal-related metabolic changes on MP numbers, cellular origin, specific subpopulations, and functional

characteristics and 3) their association with markers of vascular function. Earlier, we had shown that specific MP subpopulations, i.e. those exposing tissue factor, were elevated in patients with uncomplicated T2DM and associated with features of the MetS.[29]

In this thesis, we took these preliminary observations to the next level by using a real-life study design, by studying carefully selected, sufficiently contrasting populations and by taking a "smart-phenotyping" approach. The latter involved an integrated detailed characterization of the cardiometabolic state and the diurnal and meal-related changes thereof in all participants. Other unique and novel aspects included the state-of-the-art characterization of *ex vivo* isolated MP, obtained at various time-points through-out a 10-h or 24-h period and the possibility to link various features of the MP to the cardiometabolic changes in the participants.

Initially, in a set of proof-of-concept studies, we investigated postprandial metabolic and MP responses in healthy young males. This approach allowed us to avoid confounding of concomitant co-morbidities and to assess whether the relatively minor metabolic disturbances caused by the 2 consecutive high-fat mixed meals, that were expected to occur in young, insulin sensitive individuals, would already impact on MP and endothelial function (**Chapters 5** and **6**). In **Chapter 5** we could demonstrate that already in these healthy, well-trained males, the postprandial state, which indeed showed mild elevations of glucose, insulin and triglycerides, but all within the normal range, increased oxidative stress and promoted endothelial dysfunction. In healthy subjects, postprandially impaired FMD correlated with meal-induced hypertriglyceridemia in some,[30-32] but not in other studies.[33,34] In our study, the decrease in FMD following the second meal was not correlated to the concomitant plasma triglyceride elevations, but rather tended to associate with the meal-related increase in levels of oxidative stress markers. However, there are some aspects to be measured. Firstly, the populations studied are not readily comparable (i.e. very physically active and therefore more insulin sensitive compared to persons with a more sedentary lifestyle). Secondly, the postprandial metabolic changes found in our subjects were relatively mild, as compared to those observed in other studies.[30,31] Thirdly, the composition and consistency (liquid versus solid) of the test-meals differed in the various studies. Finally, the changes in the different parameters measured, in relation to time following meal ingestion, cannot easily be established.

Concomitantly, circulating levels of total MP (mainly consisting of platelet-, erythrocyte- and monocyte-derived MP) increased in the postprandial state, compared with fasting conditions. Previously, a single meal-induced increase in circulating endothelial cell MP, in association with postprandial metabolic changes, was published and seemed in line with our results.[35] In contrast to Ferreira,[35] however, we could not identify any MP from

endothelial cells. A possible explanation is that we only used endothelial cell-specific antibodies (CD62e, CD106 and CD144), and that the postprandial increases in CD31-positive MP reported by these authors may be in part due to elevated platelet-derived MP.[36]

By exposing negatively charged phospholipids, mainly phosphatidylserine, but also by exposing tissue factor, MP can propagate and even initiate coagulation (reviewed in **Chapter 1b**). Since the postprandial state has been associated with coagulation activation, we assessed *in vivo* coagulation variables in the fasting and the postprandial state as well as the pro-coagulant properties of MP, isolated from fasting and post-meal plasma in **Chapter 6**. Although we found distinct changes in numbers, cellular origin and phospholipid composition of MP during exposure to 2 consecutive meals in healthy subjects, this did not lead to changes in the coagulation activation *in vivo*. Our group previously showed that patients with severe meningococcal sepsis have higher levels of MP that are procoagulant,[37] in more "mild" diseases including T2DM, however, these findings were not confirmed.[27,38,39] Therefore, our finding suggests that the observed differences in (phospho-)lipid composition of cell-derived MP during fasting and meal days reflect the ability to maintain membrane homeostasis, rather than to modify coagulation. These findings confirm earlier studies showing that changes in the phospholipid composition of MP vary between cell types and depend on the activation status of the parental cell.[39-41]

In **Chapter 7**, we extended the above-mentioned findings in young healthy males by describing the postprandial changes in MP levels in middle-aged males with and without the MetS following exposure to 3 consecutive high-fat mixed meals, given during a 24-h period. We demonstrated that changes in numbers of subpopulations of MP occur in the postprandial state and associate with metabolic changes and arterial stiffness. We found that especially MP derived from erythrocytes, activated granulocytes and platelets are elevated in the MetS during 24 hours, compared to healthy males. Moreover, we confirm and extend previous findings that increased levels of specific subpopulations of MP have been associated with impaired systemic artery elasticity in healthy subjects.[42] Of interest is our finding that higher platelet-derived MP levels during the test day associates with decreased arterial stiffness support and confirm the more recent ideas that the release of MP is not necessarily solely deleterious (see also **Chapter 9**).

Of note, using the same study design, we show that elevated levels of endothelial cell-derived (CD144+) MP circulate in patients with uncomplicated T2DM and associate with postprandial metabolic derangements and impaired FMD (**Chapter 8**). Although the correlation of (presumably) endothelium-derived (CD31+/CD42-) MP with FMD was described before in fasting patients with end-stage renal failure, we extend these findings by using an antibody that is solely expressed by endothelial cells.[43] Moreover, others have

described high levels of CD144+-MP in T2DM, especially in patients with accompanying CVD-complications.[44-46] The relevance of this finding was demonstrated by Nozaki et al., who added the CD144+-MP levels to the Framingham risk model thereby not only improving risk classification, but also showing that CD144+-MP were an independent predictor of future CVD events in a high-risk population.[47]

Finally, in **Chapter 9**, recent studies reporting both deleterious as well as beneficial aspects of MP in the context of CVD risk are summarized. In the light of previously described properties of MP, together with the association between elevated numbers of MP and clinical CVD, the prevailing view is that circulating MP are harmful, contributing to CVD and risk thereof. However, the release of vesicles may protect cells from accumulation of dangerous or redundant compounds, acting as 'dust-bags', and in this manner may contribute to cellular wellbeing and even survival.

In summary, humans with MetS and T2DM, compared to healthy individuals, are characterized by prolonged and exaggerated postprandial derangements, most notably pro-atherogenic changes in the lipid profile in both these groups, which may partly result from liver steatosis, as well as additional hyperglycemia in T2DM. This postprandial dysmetabolism leads to the increased production of reactive oxygen species causing oxidative stress and functional abnormalities of the vascular endothelium at several levels, including impairment of (NO-mediated) vasoreactivity, increased coagulation and inflammation activation and post or propter the release of MP (Figure 10.1). Collectively, postprandial dysmetabolism and the associated oxidative stress may link insulin resistance and T2DM to the disproportional incidence of CVD in these high-risk populations.

In the first chapter of this thesis we reviewed the literature, available at that time, to provide a background and justification to the studies described in this thesis. In addition to **Chapter 1a** and in line with our own studies, we now review some of the more recent literature and speculate on possible therapeutic options for postprandial dysmetabolism as a CVD risk factor.

Interventions improving postprandial glucose

More than 15 observational studies involving over 100,000 subjects have been published demonstrating that elevated postprandial glucose values, even in the high nondiabetic impaired glucose tolerance (IGT) range, contribute to an approximately threefold increase in the risk of developing coronary heart disease or a CVD event.[48] The observational and therefore not causal associations have led to the hypothesis that specifically targeting postprandial glucose or glucose excursions / variability, would reduce CVD risk and

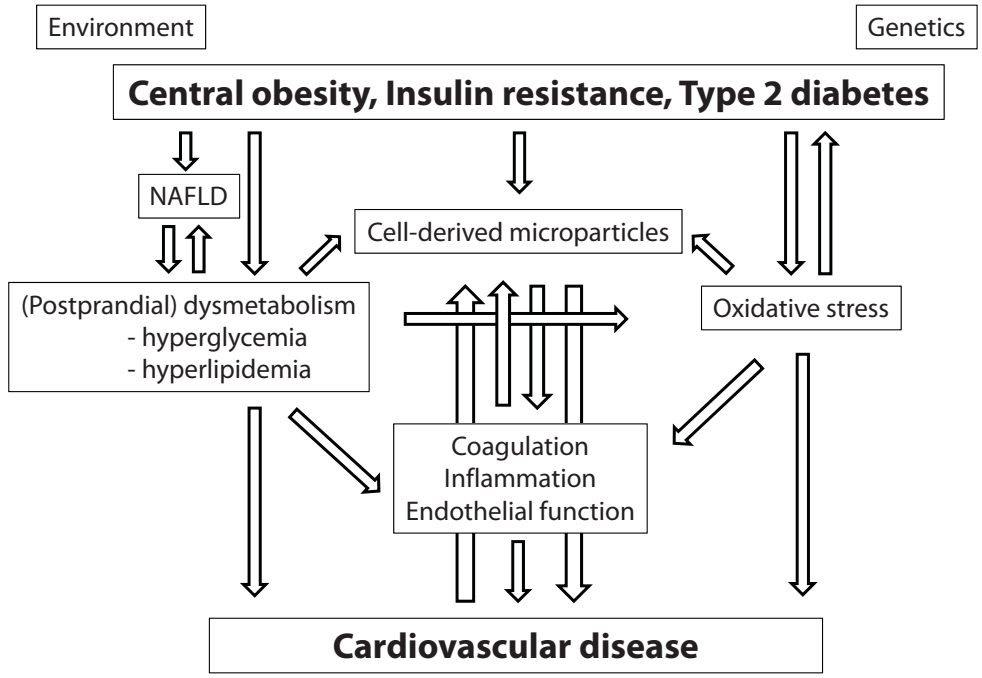

Figure 10.1 Insulin resistant states and type 2 diabetes are characterized by higher circulating numbers of specific MP, levels of atherogenic lipid particles, and oxidative stress. In the postprandial state, the numbers of MP subtypes change and the lipid abnormalities and oxidative stress are further exaggerated, with an additional adverse effect of meal-induced hyperglycemia. These postprandial derangements cause functional abnormalities of the vascular endothelium at several levels, including shedding of MP, impairment of vasoreactivity, increased coagulation and inflammation activation and increased vascular permeability. Collectively, postprandial dysmetabolism and the associated changes in MP, physiochemical properties of lipids and oxidative stress may link insulin resistance and type 2 diabetes to the disproportional incidence of CVD in these high-risk populations.

eventually also outcome. Small mechanistic studies showed that the mediating mechanisms linking glucose variability to vascular damage could be an increase in oxidative stress,[49,50] thus replicating the seminal experimental work by Michael Brownlee and co-workers.[51] However, several others were not able to reproduce these findings, suggesting that rather overall hyperglycemia, represented by HbA1c levels, should be regarded as culprit to diabetes-related complications, including macrovascular disease.[52-55]

Most established blood-glucose lowering agents lower both fasting and postprandial glucose, however, the respective effect size may differ and may depend on factors such as concomitant insulin resistance, residual beta-cell function, the extent to which glucose toxicity is lowered,

etc. To date, the few studies that indeed succeeded to preferentially lower glucose excursions have not showed benefit beyond lowering of other glycemic measures such as fasting glucose, average glucose and HbA1c.[56,57] In addition, the presumed causative role of postprandial glucose and glucose excursions as well as glucose variability, as opposed to other measures of glycemia, in the development of diabetes-related complications is still under debate.[58] In spite of this controversy, several years ago, the International Diabetes Federation has commissioned a group of experts to write guidelines addressing the link between postprandial glucose and CVD and possible therapeutic approaches.[59] Many pharmaceutical companies, however, in the quest of increasing the sales of their products, have successfully used the concept of postprandial glycemia as culprit and thus as treatment target in their marketing campaigns.

Life style

Not only do lifestyle interventions prevent or delay the onset of T2DM in high-risk populations, exercise has been shown to acutely and chronically lower (fasting and) postprandial glycemia.[60-66] Hostmark et al. demonstrated an acute blood glucose reducing effect during 30 min of bicycling following the consumption of a carbohydrate-rich meal.[65] More recently it was shown that blood glucose responses were reduced by slow postmeal walking for less than one hour.[66]

Metformin

Metformin is now established as a first-line blood glucose lowering therapy for the management of T2DM. It has also been shown to reduce the risk of to develop T2DM and reduce micro- and macrovascular complications in T2DM.[64,67,68] Although not prescribed primarily for this action, in addition to lowering fasting and mean blood glucose levels, resulting in HbA1c lowering, metformin has been shown to reduce postprandial glucose peaks,[69] possibly by indirect effects like reducing insulin resistance and hepatic fat content.[70]

Sulfonylureas

Sulfonylureas act by binding to sulfonylurea receptors on pancreatic beta-cells, leading to increased secretion of insulin with their glucose lowering potency similar to metformin.[71,72] Moreover, not only do they lower postload glucose concentrations, they have also shown to lower overall glycemia, i.e. fasting, postload and HbA1c, and have a beneficial effect on the so-called metabolic memory of glycemic legacy.[73] The most common members of this class are glibenclamide (gliburide), glipizide, gliclazide and glimepiride. The main side effects are weight gain and severe hypoglycemia, particularly in elderly people treated with long acting sulfonylureas.

Meglitinides

Meglitinide analogues are a class of oral hypoglycemic agents that increase insulin secretion, in particular, during the early phase of insulin release.[74,75] This property, along with clinical trial evidence, supports the potential role of meglitinide analogues in augmenting the early phase insulin release and, therefore, postprandial glucose control. Two analogues are currently available for clinical use: repaglinide and nateglinide, both with a rapid rise in insulin concentrations after dosing and a short half-life. Early trial evidence supports their effect in reduction of postprandial glucose and reduction in hypoglycemic episodes.[74]

Acarbose

The α-glucosidase inhibitor acarbose inhibits postprandial glucose concentrations by inhibition of α-amylase and α-glucosidase activities. Although acarbose has been shown to delay conversion to T2DM[76] and to have beneficial effects on 2-hour postload glucose concentrations, there was no improvement of beta-cell function.[77]

Thiazolidinediones

Peroxisome-proliferator activated receptors (PPAR)-γ agonists (or thiazolidinediones), such as pioglitazone and rosiglitazone, ameliorate insulin resistance, among others by decreasing lipotoxicity and stimulating growth of small insulin sensitive adipocytes, lower blood glucose and improve diabetic dyslipidemia.[78] In diabetic subjects with poor glycemic control, pioglitazone improves oral glucose tolerance mainly by enhancing the suppression of endogenous glucose production and improving beta-cell function.[79] The suppression of endogenous glucose production by pioglitazone is closely related to the reduction in hepatic fat content.[80]

Insulins

There is evidence to show that patients who added a prandial insulin-based regimen to oral therapy have better glycated hemoglobin (HbA1c) than patients who add a basal insulin-based regimen.[81]

Pramlintide

Pramlintide is a synthetic analogue of amylin, a neuroendocrine hormone that is co-secreted with insulin from pancreatic beta-cells in response to nutrient intake.[82] It plays an important role in the maintenance of postprandial glucose homeostasis by regulating the rate of glucose appearance into the circulation through three primary mechanisms:

regulation of gastric emptying, reduction of postprandial glucagon secretion, and regulation of food intake. Through these same mechanisms, pramlintide significantly reduces postprandial glucose concentrations when used as an adjunct to mealtime insulin in both T1DM and T2DM.[83,84] To date, there are no outcome studies reported for pramlintide.

Incretin-based therapies

Glucagon-like peptide 1 (GLP-1) is a gut-derived incretin hormone that, in a glucose-dependent manner, stimulates meal-related insulin secretion (and production) and suppresses glucagon secretion, slows down gastric emptying, and reduces appetite and food intake.[85] In a 6-week intervention study, in which GLP-1 was administered continuously via a subcutaneous insulin-pump delivery system in T2DM patients, the incretin hormone was shown to reduce body weight.[86] Thus, since GLP-1 addresses several of the pathophysiological defects of the T2DM phenotype, it was regarded as an ideal approach for the treatment of T2DM. However, native GLP-1 is readily degraded by the ubiquitous enzyme dipeptidyl peptidase-4 (DPP-4) and consequently has a circulating half-life time of 1-2 minutes. Therefore, two different strategies were followed in order to enhance incretin action and harness GLP-1 as a therapeutic option for T2DM, including the development of injectable degradation-resistant GLP-1 receptor agonists (GLP-1RA; e.g. exenatide, liraglutide) and oral inhibitors of DPP-4 activity (DDP-4i; e.g. sitagliptin, vildagliptin and saxagliptin). All of these incretin-based therapies have been shown to decrease postprandial glucose concentrations.[87-89]

Interventions improving postprandial lipemia

Since the pro-atherogenic postprandial involves both glucose and lipids and their interaction, (see also **Chapter 1a**), therapeutic strategies that affect plasma glucose levels, may also influence postprandial lipids and lipoproteins.

Life style

There is a large body of evidence showing that postprandial lipemia can be attenuated by exercise, both before and following a meal.[90-95] For example, for physically inactive individuals with the MetS, exercising at moderate intensity for 45 min attenuates postprandial hypertriglyceridemia and improve insulin action.[95]

Fibrates – statins

Fibrates, drugs in the class of amphiphatic carboxylic acids, have multiple blood lipid modifying actions due to their ability to bind and activate the transcription factor PPARα. Treatment with fibrates did not only show a reduction in postprandial hypertriglyceridemia, also the postprandial increase of larger-sized VLDL and smaller-sized LDL particles, oxidized fatty acids and oxLDL was abrogated by fenofibrate.[96-101] Recently, the triglyceride lowering actions in the postprandial state of fenofibrate include alterations in triglyceride and fatty acid metabolism in the small intestine of high-fat fed mice.[101]

The most important action of 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) have been proved highly effective in reducing CVD event risk, both in primary and secondary prevention trials.[102-104] Moreover, statins have been shown to reduce postprandial lipemia,[96,105-107] although not as effective as fibrates.[100]

Metformin

Although mainly known for improving glycemic control in T2DM, metformin also has favorable effects on postprandial lipid metabolism.[108-110] Moreover, in non-obese T2DM patients, fasting and integrated 6-h postprandial measures of plasma glucose, cholesterol, triglycerides and serum FFA decreased equally after 4 months of metformin versus repaglinide treatment.[110]

Sulfonylureas

Data on the effect of sulfonylureas on postprandial lipid metabolism are inconsistent and conflicting.[111-116] However, most of the studies suggest that sulfonylureas improve postprandial lipemia ameliorating the postprandial increase in FFAs, chylomicrons and VLDL triglycerides. This effect is possibly due to the increased activity of lipoprotein lipase (LPL) and hepatic lipase (HL) by hyperinsulinemia and to the reduction of glycemia and FFAs postprandially.

Meglitinides

Although meglitinides increase postprandial insulin secretion and decrease postprandial glycemia, most of the available studies show a rather disappointing impact on postprandial lipemia in patients with T2DM.[116-118]

Acarbose

Already in the late 1970s the (dose-dependent) effectiveness of acarbose in the reduction of postprandial hypertriglyceridemia was shown.[119] More recently, it was demonstrated that acarbose attenuates the postprandial increase of total triglycerides, chylomicrons, VLDL and lipoprotein remnants concentrations.[120-122] It may contribute to the regulation of LPL activity by modifying apoC-II and apoC-III levels.

Thiazolidinediones

Remarkable differences exists between the effects of rosiglitazone and pioglitazone on postprandial lipemia in several study populations.[80,115,123-128] Despite metabolic improvements (insulin sensitivity and FFA handling), rosiglitazone caused no significant effects on fasting and postprandial triglycerides,[123-126] and even showed a marked increase in postprandial remnant like cholesterol particles in patients with HIV-lipodystrophy.[126] In contrast, pioglitazone has been shown to decrease insulin sensitivity and favorably modify fasting and postprandial triglycerides, FFAs and HDL cholesterol in overweight and obese non-diabetic patients with coronary artery disease and T2DM patients.[80,115,128] In addition, pioglitazone has been proven to improve NAFLD and hitherto has beneficial effects on postprandial lipidemia and glycemia.[80,128]

Insulins

Clinical studies have shown that insulin treatment in patients with T2DM is associated with a less (postprandial) atherogenic lipid profile, an effect independently of blood glucose control.[129-133] Rapid acting insulin analogues have been shown to have more favorable effects on postprandial lipemia than longer acting insulins.[132,133]

Incretin-based therapies

The short-acting GLP-1 receptor agonist exenatide (administered twice daily before breakfast and dinner) significantly lowered postprandial lipidemia in metformin-treated patients with T2DM, as compared to insulin glargine or placebo.[88,89,134]

Initially, the postprandial lipid-lowering effects were attributed to the concomitant slowing of gastric emptying, but recent studies in humans and animals have shown that the incretin hormones may directly interfere with lipoprotein metabolism, independently of the effects on gastric emptying.[135-137] Thus, short time treatment (4 weeks) with the DPP-4i vildagliptin, which does not affect gut motility, improved postprandial plasma triglyceride and apolipoprotein B-48-containing triglyceride-rich lipoprotein particle metabolism in T2DM patients after a fat-rich meal.[136] Similar results were obtained in a 6-week study

of sitagliptin versus placebo.[137] The direct effects of GLP-1 on lipoprotein assembly were demonstrated in a series of elegant preclinical experiments, in which, among others, the GLP-1R agonist exendin-4 directly reduced Apo48 secretion in enterocyte cultures from fructose-fed hamsters.[135] Taken together, these novel agents are regarded as very promising, as they not only reduce blood glucose, but also have a potential to lower the cardiovascular risk profile in patients with T2DM.

Interventions lowering postprandial oxidative stress

Oxidative stress, in particular the increased superoxide production at the mitochondrial level, has been suggested as the key link between postprandial dysmetabolism and diabetes complications.[138] Not only do postprandial hyperglycemia and hyperlipidemia separately increase oxidative stress, also evidence for a cumulative effect of both is present.[139] Both acute and chronic exercise may aid in attenuating postprandial oxidative stress by improving blood glucose and triglyceride clearance, and by stimulating an increase in endogenous antioxidant enzyme activity.[91-93,140] The effect of blood-glucose lowering drugs, including metformin, sulfonylureas, acarbose and insulin, on the amelioration of postprandial oxidative stress and endothelial function are not consistent.[83,84,129,141,142] The effect may be mainly due to their long-term glucose-lowering actions and the concomitant alleviation of oxidative stress.138Aside their lipid lowering potency, statins have been shown to reduce postprandial oxidative stress and improve endothelial function.[96,105,106,139,143,144] Also fibrates showed postprandial oxidative stress reduction, although to a lesser extent than statins.[97,105] Previously, in a small intervention study, a attenuating effect of pioglitazone on oxidative stress following a meal test was described.[115] Not only did exenatide (administered twice daily before breakfast and dinner) significantly lowered postprandial glycemia and lipidemia in metformin-treated patients with T2DM, these effects were related to changes in the oxidative stress markers as compared to insulin glargine[88] and placebo.[89] The use of anti-oxidants and vitamins show promising results in short-term postprandial studies assessing the effect on endothelial function and markers of oxidative stress.[145-147]

Interventions interfering with (postprandial) MP formation

Various anti-platelet drugs, including the GPIIb/IIIa receptor antagonist abciximab[148] and the cAMP phosphodiesterase inhibitor cilostazol,[149] offer therapeutic possibilities, as they reduce excessive platelet-derived MP formation. Most notably, there was no influence of prior exercise on postprandial circulating levels of presumed endothelial MP (CD31+/42b-), despite considerable reduction in lipemia.[150] Recently, it was demonstrated that mitiglide lowers baseline levels of platelet-derived MP.[151] From the same group, a comparable effect of

acarbose was shown in T2DM patients following three months of treatment.[152] In contrast, previously we showed *in vitro* that simvastatin facilitated the release of MP by endothelial cells.[153] In a small pilot study, pioglitazone decreased the number of circulating endothelial MP independently of plasma glucose and lipid concentrations in subjects with the MetS.[154] The effect of pioglitazone or GLP-1 treatment on postprandial MP levels is not known. Short-term administration of vitamin C at a high dose reduced the number of circulating endothelial-cell-derived MP in patients with congestive heart failure.[155] Moreover, the possible beneficial effect of anti-oxidants was previously demonstrated by an anecdotal observation, in which consumption of a flavinoid-rich cocoa beverage reduced circulating numbers of platelet-derived MP in healthy subjects.[156]

What is the evidence of these interventions to reduce CVD risk?

The cheapest and probably best intervention to reduce CVD and other chronic disease risk is change of life style and additional weight loss.[61-64,157-161] However, since most of the adults have adapted their life style for several years, a beneficial change in their habits for a longer period of time seems far too difficult without cognitive behavioral therapy.[161]

The fastest, most effective and durable therapy to lose a large amount of weight within hours is by bariatric surgery. Although this is accompanied by serious postoperative complications, it leads to reduction, or even remission, of T2DM and CVD risk.[162,163]

Several controlled, prospective, and randomized clinical trials, e.g. the STOP-NIDDM trial, the NAVIGATOR trial, and the HEART2D trial have not proofed that targeting postprandial hyperglycemia results in a more beneficial outcome of CVD complications in IGT subjects or overt type 2 diabetic patients.[164-167] A possible explanation is the inclusion of patients with a high CVD risk and long-term T2DM duration, who had already accumulated considerable cardiovascular compromise. The use of anti-oxidants and vitamins may show promising acute effect on postprandial endothelial dysfunction and markers of oxidative stress.[145-147] However, so far, the use of anti-oxidants and vitamins has not yielded the expected benefit in long-term prospective trials.[168,169]

The role of statins in lowering CVD risk seems undisputable. Large studies demonstrated lowering of LDL cholesterol and C-reactive protein concentrations, and increase of LDL size and HDL cholesterol concentration, accompanied with a reduction of CVD events.[102-104,170] Of interest, however, a meta-analysis of mega trials suggested that, with the exception of pravastatin, the statins appear to modestly increase the risk of new diabetes.[171] In prospective placebo-controlled studies of T2DM patients, fibrates have been shown to be inconsistent in their ability to decrease cardiac events.[172-174] In the Helsinki Heart study, cardiac events

were decreased by 71% in the group of patients who had all the characteristics of the MetS, compared to controls.[172] In a subgroup analysis of the VA-HIT trial, treatment with gemfibrozil resulted in a more than 30% reduction of cardiac events in patients with T2DM and in those with insulin resistance.[173] In contrast, daily administration of fenofibrate did not change the primary endpoint of fatal myocardial infarction or death related to CVD in the FIELD study. However, there was a significant 24% reduction in non-fatal myocardial infarction and 11% decrease in CVD events.[174]

Recently, rosiglitazone was associated with a significant increase in the risk of myocardial infarction and has been withdrawn from the market.[175] In contrast, the PROactive trial of the thiazolidinedione, pioglitazone, found no benefit with regard to its primary aggregate cardiovascular outcome, but it did find a 16% decrease in its secondary end point – death, myocardial infarction, and stroke – after three years of treatment.[176] However, recently an association of pioglitazone use and a higher risk of developing bladder cancer has been described.[177,178] Presently, several long-term outcome studies for both GLP-1RA and DPP-4i are underway that will hopefully demonstrate their added value in the treatment of T2DM and its (CVD) complications.[179-181] Initial meta-analysis of phase 2 and 3 studies of these agents are promising and at least did not show any indication of harm.[182-184] Moreover, treatment with exenatide showed a hazard ratio of 0.81 [95%CI 0.68-0.95] for CVD events.[182]

FUTURE PERSPECTIVES

Realizing that today the role of fasting versus postprandial hyperglycemia and hyperlipidemia as CVD risk factors (versus risk markers) remains unresolved, we need future studies to establish a causal relationship between oscillating versus more stable glucose, lipid levels and their physiochemical properties, and cardiovascular damage. Subsequently, the mechanisms that could explain the causal link need to be addressed. The role of MP numbers, their cellular origin, composition and their role in cell survival and coping with (meal-induced) stress, and eventually atherogenesis has become less clear, their presence being not solely deleterious. Furthermore, assuming that oxidative stress appears to be the key mechanism underlying all the phenomena reported above, long-term therapeutic options that reduce the associated CVD are still lacking. GLP-1 receptor agonists show promising results in recent studies, although cardiovascular outcome studies are still underway.

A noteworthy recent development is the increasing interest in a role for non-digestible carbohydrates, short-chain fatty acids, prebiotics and antibiotics in manipulation of gut microbiota, affecting energy harvesting from gut, obesity, incretin levels, glucose intolerance

and the proinflammatory response.[185,186] Of interest could be the role of gut microbiota, nutrients and functional foods as well as the above-mentioned pharmaceutical treatments in postprandial dysmetabolism, MP, oxidative stress and the risk of developing CVD. As described, many options are open, which warrants substantial future improvement in treatment of MetS, T2DM and ultimately avoidance of CVD.

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11

Samenvatting voor de geïnteresseerde leek

Het voorkomen van (ernstig) overgewicht of obesitas neemt wereldwijd ernstige vormen aan, met name door het overnemen van de zogenaamde Westerse leefstijl, dat wil zeggen hoge calorie-inname en weinig lichamelijke beweging. Deze verandering in leefstijl met bijkomend overgewicht leidt tot ongevoeligheid voor het hormoon insuline, een hormoon dat onder andere een belangrijke rol speelt bij de suiker- en vetstofwisseling. Door deze ongevoeligheid of resistentie kan insuline, dat dus wel aanwezig is, niet goed zijn werking hebben. Dit leidt tot hoge concentraties van insuline in het bloed, verhoogde bloeddruk en een gestoord vet- of lipidenmetabolisme, allemaal bekende risicofactoren voor het ontwikkelen van hart- en vaatziekten. Deze combinatie van factoren wordt het metabool syndroom genoemd en dit syndroom speelt een belangrijke rol bij het ontwikkelen van type 2 diabetes mellitus, (voorheen ook wel 'ouderdomssuikerziekte' genoemd). Bij personen die hier namelijk een genetische aanleg voor hebben, kan de voortdurende vraag naar insuline resulteren in het tekort schieten van de insuline-producerende cellen in de alvleesklier, of te wel de betacellen. Hierdoor ontstaat onder andere een (te) hoge bloedsuiker en dus type 2 diabetes mellitus.

Bij mensen die insuline-ongevoelig zijn en type 2 diabetes mellitus hebben, zullen de stofwisselingsstoornissen meer uitgesproken worden wanneer zij een maaltijd eten. Hierdoor ontstaan tijdelijk nog hogere concentraties van suiker (glucose), vetdeeltjes (triglyceriden en vrije vetzuren) en insuline in het bloed. Omdat het langer duurt voordat deze verstoringen verwerkt zijn en men meerdere maaltijden per dag eet, zijn de bloedvaten bijna 24 uur per dag blootgesteld aan een milieu waarin gemakkelijk ontsteking, bloedstolling en (daardoor) aderverkalking (atherosclerose) ontstaan.

Patiënten met type 2 diabetes hebben een sterk verhoogd risico op het ontwikkelen van hart- en vaatziekten. De zogenaamde 'klassieke' risicofactoren hiervoor zijn roken, hoge bloeddruk en hoog cholesterolgehalte. Deze factoren kunnen echter het hoge risico voor hart- en vaatziekten bij patiënten met type 2 diabetes maar deels verklaren. Het is niet ondenkbaar dat de stofwisselingsstoornissen die optreden na de maaltijd, ofwel postprandiaal, leiden tot verstoringen aan de bloedvatwand en bijdragen aan het ontstaan van hart- en vaatziekten. Aanwijzingen voor deze relatie worden in **Hoofdstuk 1a** uitgebreid besproken en samengevat aan de hand van de literatuur.

In dit proefschrift hebben we de relatie tussen postprandiale verstoringen in onder andere glucose, insuline en vetdeeltjes, en het risico op het ontstaan van hart- en vaatziekten bestudeerd bij mensen met een verhoogd risico hierop. Verder hebben we mogelijke onderliggende mechanismen, zoals toename van oxidatieve stress, verstoring van functie van de vaatwandbekleding (endotheel) en veranderingen in aantallen en samenstelling van zogenaamde micropartikels, na het nuttigen van maaltijden bestudeerd.

Micropartikels zijn blaasjes die afgesnoerd worden door diverse cellen in lichaamsvloeistoffen, zoals bloed en speeksel, tijdens activatie en celdood (apoptose). Deze micropartikels vormen een heterogene populatie en hun aantal, cellulaire herkomst, samenstelling en functionele eigenschappen hangen nauw samen met de omstandigheden die tot hun ontstaan hebben geleid. In het bloed van gezonde personen zijn micropartikels aanwezig die met name afkomstig zijn van bloedplaatjes (trombocyten), maar in geringere mate ook van witte bloedcellen (lymfocyten, leukocyten), rode bloedcellen (erytrocyten) en endotheelcellen. De stolling, veruit de bekendste eigenschap van micropartikels, wordt gefaciliteerd door de aanwezigheid van negatief geladen fosfolipiden op het micropartikel-oppervlak en, soms, door de expositie van weefselfactor. Daarnaast zijn micropartikels ook betrokken bij ontstekingsreacties, het moduleren van endotheelfuncties, het bevorderen van celgroei, chemotaxis, apoptose en het uitgroeien van getransplanteerde hematopoëtische stamcellen. Op grond van hun functionele eigenschappen vormen micropartikels mogelijk de ontbrekende schakel tussen de verschillende processen die ten grondslag liggen aan het ontstaan van atherosclerotische vaatschade. In **Hoofdstuk 1b** bespreken we uitgebreid de mogelijke rol van micropartikels bij het ontstaan van hart- en vaatziekten.

Voorgaande onderzoeken waarbij het effect van een maaltijd op risicofactoren voor het ontstaan van hart- en vaatziekten werd gemeten, maakten gebruik van slechts 1 maaltijd, vaak vloeibaar van samenstelling, waarna vervolgens tot 8 uur allerlei metingen werden verricht. Dit terwijl men in het dagelijks leven meerdere (vaste) maaltijden per dag nuttigt. Om eerder genoemde mogelijke relatie tussen verschillende componenten van postprandiale metabole verstoringen en risicofactoren voor het ontwikkelen van hart- en vaatziekten te bestuderen, kozen wij vervolgens voor een uitgebreide onderzoeksopzet. Om de rol van hoge bloedglucosewaarden te ontrafelen, hebben we metingen verricht bij mannen met het metabool syndroom (overgewicht, verhoogde bloeddruk, gestoorde vetstofwisseling) zonder gestoorde bloedglucosewaarden en bij mannen die dat wel hadden, dus type 2 diabetes mellitus. Om vervolgens de bijdrage van het metabool syndroom te meten, hebben we beide groepen vergeleken met gezonde mannen van gelijke leeftijd. Door deze mannen gedurende 24 uur te observeren, waarbij zij 3 vaste gestandaardiseerde maaltijden kregen voorgeschoteld als ontbijt, lunch en avondeten op vaste tijdstippen, konden wij diverse aspecten van onder andere het vetmetabolisme bij deze groepen deelnemers bestuderen. Het is mogelijk dat de hoeveelheid vet in de lever hierbij een belangrijke rol speelt omdat de lever van belang is voor de vetstofwisseling en een vervette lever, de zogenaamde Non-Alcoholic Fatty Liver Disease (NAFLD), vaak voorkomt bij mensen met het metabool syndroom en type 2 diabetes. In **Hoofdstuk 2** laten we zien dat meerdere maaltijden, genuttigd als ontbijt, lunch en avondeten, bij

mannen met het metabool syndroom en met type 2 diabetes, inderdaad leidt tot hogere concentraties van vetdeeltjes in het bloed, vergeleken met gezonde mannen. De hoeveelheid vet in de lever blijkt hierin van belang te zijn: hoe meer levervet, hoe hoger de verstoringen waren. Daarnaast beschrijven we in **Hoofdstuk 4** dat de hoeveelheid levervet effect heeft op de verzadiging van HDL-cholesteroldeeltjes (het zogenaamde 'goede cholesterol') met vetdeeltjes na 3 maaltijden, waardoor deze HDL-cholesteroldeeltjes minder goed functioneren. Niet alleen vonden we dat de functie van het HDL-cholesterol verstoord raakte, ook een functie van de binnenbekleding van de vaatwand, het endotheel, werd gestoord na het nuttigen van de 3 maaltijden, vooral bij de mannen met type 2 diabetes.

De postprandiale veranderingen in HDL- en LDL (het 'slechte' cholesterol)-cholesteroldeeltjes hebben we nader bestudeerd. De resultaten hiervan staan beschreven in **Hoofdstuk 3**. We laten zien dat de (langdurig) verhoogde concentraties van vetdeeltjes in het bloed na de maaltijd, zoals we zien bij het metabool syndroom en type 2 diabetes, resulteert in verzadiging van de HDL- en LDL-deeltjes, waardoor deze ontvankelijk worden voor oxidatie en hierdoor kunnen bijdragen aan het ontstaan van hart- en vaatziekten.

Ten tijde van het opstarten van het onderzoek was nog niet bekend wat het effect is van postprandiale veranderingen op de vorming en samenstelling van micropartikels. Daarom hebben wij allereerst bij gezonde jonge mannen uitgezocht wat het effect is van 2 gestandaardiseerde, vaste en opeenvolgende maaltijden (ontbijt en lunch) op deze celblaasjes in combinatie met eerdergenoemde risicofactoren, en vergeleken we dit met de resultaten van een dag waarop de deelnemers nuchter bleven. In **Hoofdstuk 5** beschrijven we dat bij deze gezonde jonge mannen de veranderingen die optreden in de concentraties van glucose, vetdeeltjes en insuline in het bloed na inname van 2 vetrijke maaltijden, al leiden tot verhoogde oxidatieve stress, disfunctioneren van het endotheel en verhoogde aantallen micropartikels, in vergelijking met de nuchtere dag.

Omdat de postprandiale periode en micropartikels beiden in relatie worden gebracht met de bloedstolling, hebben we bestudeerd of de gevonden veranderingen in aantallen micropartikels gerelateerd waren aan bloedstolling. Deze resultaten worden beschreven in **Hoofdstuk 6**. Niet alleen bestudeerden we de aantallen micropartikels, maar ook hun cellulaire herkomst, samenstelling en eigenschap om bloedstolling in het laboratorium te bevorderen. Hierbij vonden we dat in de postprandiale periode vooral het aantal micropartikels afkomstig van rode bloedcellen verhoogd raakt. Daarnaast zagen we dat er veranderingen optraden in de samenstelling van de wand van de micropartikels, namelijk van de zogenaamde fosfolipiden, waaruit de wand voor een groot deel bestaat. Ondanks de verhoogde aantallen en veranderingen in de fosfolipidensamenstelling zagen we geen verandering in de bloedstolling, zowel in het bloed van de deelnemers tijdens de testdagen

als bij het bestuderen van de stollingsbevorderende eigenschappen van de micropartikels in het laboratorium.

In **Hoofdstuk 7** beschrijven we het effect van de metabole verstoringen die ontstaan na het nuttigen van 3 maaltijden (ontbijt, lunch en avondeten) op aantallen en herkomst van micropartikels bij mannen van middelbare leeftijd met en zonder het metabool syndroom. Verder onderzochten we of deze eventuele veranderingen gerelateerd waren aan de stijfheid van de bloedvaten. De stijfheid van de bloedvaten is een risicofactor voor het ontstaan van hart- en vaatziekten: hoe stijver de bloedvatwand, hoe hoger het risico. We laten zien dat er veranderingen in de aantallen micropartikels van rode bloedcellen en van geactiveerde witte bloedcellen en bloedplaatjes optraden bij de mannen met het metabool syndroom. Opvallend is dat deze bepaalde subpopulaties zowel positief als negatief gerelateerd waren aan de stijfheid van de bloedvaten. Zo waren hogere aantallen van bloedplaatjes afkomstige micropartikels gerelateerd aan een verminderde stijfheid van de bloedvaten. Deze bevindingen passen bij de gedachte dat micropartikels niet alleen nadelige gevolgen hebben.

Bij mannen met type 2 diabetes vonden we dat de metabole verstoringen die optraden na het eten van de 3 maaltijden leidden tot verhoogde aantallen van micropartikels afkomstig van de binnenbekleding van bloedvaten (endotheel). Niet alleen waren deze aantallen gerelateerd aan de hoogte van de vetdeeltjes-, glucose- en insulineconcentraties, ook hingen deze aantallen micropartikels samen met een functie van het endotheel, namelijk het zogenaamde vaatverwijdende vermogen hiervan. Deze resultaten staan beschreven in **Hoofdstuk 8**.

In **Hoofdstuk 9** gaan we uitvoerig in op de rol die micropartikels zouden kunnen spelen in het ontstaan van hart- en vaatziekten. We beschrijven dat micropartikels niet alleen nadelige gevolgen kunnen hebben, maar zelfs ook gunstige gevolgen. Het afsnoeren van micropartikels door cellen zou beschouwd kunnen worden als het afgeven van vuilniszakjes waardoor de cel gevrijwaard blijft van schadelijke prikkels afkomstig van zowel binnen als van buiten de cel. Hierdoor draagt de afsnoering van micropartikels bij aan de vitaliteit en overleving van de cel.

Samenvattend, bij mannen met het metabool syndroom en mannen met type 2 diabetes wordt de postprandiale staat gekarakteriseerd door verhoogde concentraties van schadelijke vetdeeltjes in het bloed, die vervolgens ook daarin langer blijven circuleren vergeleken met gezonde mannen. Dit wordt voor een deel verklaard door de verhoogde hoeveelheid vet in de lever, voor een ander deel door verhoogde glucoseconcentraties bij type 2 diabetes. Deze postprandiale metabole verstoringen leiden tot verhoogde oxidatieve stress, verstoringen

van de functie van het endotheel en de afsnoering van micropartikels door verschillende cellen. Hierdoor zouden verstoringen in het postprandiale metabolisme bij kunnen dragen aan het ontstaan van hart- en vaatziekten, vooral bij populaties met een verhoogd risico hierop zoals mensen met het metabool syndroom en type 2 diabetes.

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Del concerto della vita nessuno ottiene un programma

Curriculum vitae

Martinus Everardus ('Maarten') Tushuizen was born on September 3rd, 1974 at the Free University Hospital in Amsterdam, The Netherlands. After graduation from secondary school (pre-university education, Geert Groote School, Amsterdam) in 1993, he did not pass the numerus clausus for Medicine and started studying Medical Biology at the Free University, Amsterdam and obtained his propaedeutic diploma in 1994. After a year he could switch to medicine at the Free University and graduated as a medical doctor (MD) in July 2001. From September 2001 until December 2006, he was a PhD student at the Diabetes Center at the Department of Internal Medicine (than Department of Endocrinology) of the VU University Medical Center in Amsterdam, under supervision of Prof. Michaela Diamant, in collaboration with the Laboratory of Experimental Clinical Chemistry of the Academic Medical Center in Amsterdam, under supervision of Prof. Auguest Sturk and Dr. Rienk Nieuwland. Most of the results of these years of research are described in this thesis. He started his training in Internal Medicine in January 2007 at the Rijnland Hospital in Leiderdorp, under supervision of Dr. Frans Cluitmans and Dr. Martien Janssen, affiliated with Leiden University Medical Center, Leiden, The Netherlands. In January 2009 he switched specialty training and started training in Gastroenterology and Hepatology at Medical Center Alkmaar in Alkmaar under supervision of Dr. Hans Tuynman, affiliated with the VU University Medical Center. In 2010 he continued his training at the Department of Gastroenterology and Hepatology at the VU University Medical Center, supervised by Prof. Chris Mulder. During his training he was visiting fellow/specialty registrar Hepatology at the Erasmus Medical Center, Rotterdam, The Netherlands and the Queen Elizabeth University Medical Centre, Birmingham, United Kingdom. Maarten is married with Fleur and together they have two children (Tom and Julianne) and are expecting a third.

Martinus Everardus ('Maarten') Tushuizen werd geboren op 3 september 1974 in het VU Ziekenhuis te Amsterdam. Na het afronden van de middelbare school in 1993 (VWO, Vrije School Geert Groote te Amsterdam), werd hij uitgeloot voor de studie Geneeskunde en ging hij Medische Biologie studeren aan de Vrije Universiteit te Amsterdam. Na het behalen van zijn propedeuse in 1994 werd hij ingeloot voor de studie Geneeskunde aan dezelfde universiteit en behaalde in juli 2001 zijn artsexamen. Van september 2001 tot januari 2007 was hij arts-onderzoeker bij het Diabetescentrum van de afdeling Interne Geneeskunde (aanvankelijk afdeling Endocrinologie) van het VU medisch centrum, onder leiding van Prof. Dr. M. Diamant en in samenwerking met het Laboratorium van Experimentele Klinische Chemie van het Academisch Medisch Centrum te Amsterdam, onder leiding van Prof. Dr. A. Sturk en Dr. R. Nieuwland. Een groot deel van het verrichte onderzoek is beschreven in dit proefschrift. In januari 2007 begon hij als internist in opleiding in het Rijnland Ziekenhuis te Leiderdorp met als opleiders Dr. F.H. Cluitmans en Dr. M.J. Janssen, geaffilieerd aan het Leids Universitair Medisch Centrum te Leiden. In januari 2009 wisselde hij van opleiding en begon als maag-, darm- en lever(MDL)-arts in opleiding in het Medisch Centrum Alkmaar te Alkmaar, met als opleider H.A. Tuynman, geaffilieerd aan het VU medisch centrum te Amsterdam. In 2010 vervolgde hij zijn opleiding in het VU medisch centrum met als opleider Prof. Dr. C.J. Mulder. Gedurende zijn opleiding volgde hij stages Hepatologie in het Erasmus Medisch Centrum te Rotterdam en het Queen Elizabeth University Medical Centre te Birmingham, Verenigd Koninkrijk. Maarten is getrouwd met Fleur en zij hebben samen twee kinderen (Tom en Julianne) en zijn in verwachting van een derde.

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