General discussion and future perspectives

The prevalence of obesity is reaching pandemic proportions, mainly because of adopting 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 the metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM), i.e. insulin resistance with concomitant central obesity, hyperinsulinemia, hypertension and dyslipidemia, all established risk factors for the development of cardiovascular disease (CVD).² 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 failure of the beta-cells 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.³,⁴ Collectively, these derangements result in systemic and vascular inflammation, impaired endothelial function and ultimately, in CVD.⁵,⁶ Besides, 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.⁷-⁹
In this thesis, it is attempted to obtain a more detailed understanding of the previously reported relationship between the postprandial state, i.e. hyperglycemia, hyperlipidemia and hyperinsulinemia, 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. 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, as well as different lipid profiles and, importantly, care should be taken that all are postmenopausal to avoid further confounding of different hormonal states. 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 12-h or 24-h study period during which the participants were given 2 (12-h period), respectively 3 (24-h period) consecutive standardized high-fat mixed meals, given as breakfast and lunch or as breakfast, lunch and dinner.

**Major findings**

This thesis in fact has two parts, i.e. the first part in which we focus on postprandial changes in the pro-atherogenic lipid profile in men with MetS and in those with T2DM, as compared
to controls, and attempt to establish a link between the consequences, including markers of oxidative stress and inflammation as well as vascular (functional) changes on the one hand, but also to factors leading to these postprandial derangements, such as liver fat accumulation (Chapters 2-4). In the second part, we detail the differences in numbers, cellular origin, subpopulations and functional characteristics of MP among healthy individuals and those with MetS and T2DM (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. 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, 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 postprandial pro-atherogenic changes in the 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). 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 alters the anti-oxidative capacity of this lipoprotein class, which is paralleled by endothelial dysfunction measured by ultrasound as flow mediated dilatation (FMD). Our findings are in line with and 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.\textsuperscript{13}

The pro-atherosclerotic functional changes in HDL and LDL particles during the postprandial state were more extensively studied in Chapter 3. We showed that prolonged hypertriglyceridemia, as observed in males with the MetS and patients with T2DM following three 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 (85-g fat),\textsuperscript{14} and extend these findings by demonstrating 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.\textsuperscript{15} As described previously, Patel and coworkers demonstrated an impaired anti-inflammatory capacity of HDL in response to 20% Intralipid.\textsuperscript{13} 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 result 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 common 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 suggested alanine aminotransferase (ALT) as a marker of NAFLD and demonstrated in the population-based Hoorn Study 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.16,17 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, proper identification of patients with NAFLD seems of major importance for adequate treatment and 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.\textsuperscript{18-20} However, most human studies have focused on quantitative (circulating numbers) rather than qualitative aspects of MP, mostly of platelet origin, using 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 microparticles 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.\textsuperscript{21} In this thesis we take these preliminary observations to the next level by using a real-life study design, with carefully screened, sufficiently contrasting populations and the extensive “smart-phenotyping” approach, in which an integrated detailed characterization of the cardiometabolic state and diurnal and meal-related changes thereof were performed in all participants. Further important assets were the state-of-the-art characterization of ex vivo isolated MP, obtained at various time-points through-out a 12-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 two consecutive high-fat mixed meals, that were expected to occur in young, insulin sensitive individuals, would already impact on MP and endothelial function (\textit{Chapters 5 and 6}). In \textit{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, but not in other studies. 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. 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. In contrast to Ferreira, 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.

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 could detect changes in number, cellular origin and phospholipid composition of MP during exposure to two 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.\textsuperscript{29} in more “mild” diseases including T2DM, however, these findings were not confirmed.\textsuperscript{19,30,31} 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.\textsuperscript{31-33}

In Chapter 7, we extended previous 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 three 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.\textsuperscript{34} 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.

Of interest is that, 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. Moreover, others have described high levels of CD144+-MP in T2DM, especially in patients with accompanying CVD-complications. Of interest, the addition of CD144+-MP 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.

Finally, in Chapter 9, recent studies revealing both deleterious as well as beneficial effects of MP in the development of CVD are summarized. In the light of the previous 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, MP 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. 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, the 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. 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.

**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 and lipid levels 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 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 generation appears to be the key player of 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 long-term follow-up is absent. A noteworthy 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 thereby energy harvesting from gut, obesity, incretin levels, glucose intolerance and the proinflammatory response. Of interest could be the role of gut microbiota, nutrients and functional foods 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.
References


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