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MICROVESICLES, SKIN MICROCIRCULATION AND CLINICAL MICROANGIOPATHY IN TYPE 1 DIABETES

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Microvesicles, skin microcirculation and clinical microangiopathy in type 1 diabetes

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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In loving memory of my mum, for your kind heart, for always believing in me and for giving me the best possible foundation to build on. Without you none of this would have been possible.

Man's mind, once stretched by a new idea, never regains its original dimensions.

– Oliver Wendell Holmes

POPULÄRVETENSKAPLIG SAMMANFATTNING

Typ 1 diabetes är en livslång autoimmun sjukdom där bukspottkörteln förlorar sin förmåga att reglera blodsockret. Trots insulinbehandling drabbas många av patienterna av **småkärlssjuka** (**mikroangiopati)** i ung ålder, vilket kan ge skador på njurar (**nefropati**), ögonbotten (**retinopati**) och nerver (**neuropati**). En stor andel utvecklar nedsatt njurfunktion, synpåverkan eller kronisk smärta. Mikroangiopati bidrar även till att patienter med typ 1 diabetes löper en kraftigt förhöjd risk att drabbas av hjärt-kärlsjukdom.

Mikrovesikler (MVs) är små membranomslutna ”blåsor” som frisläpps ifrån alla celler i blodbanan och som spelar en viktig roll som budbärare i cellernas kommunikation. MVs kan bidra till inflammation, ge upphov till skador på blodkärlens insida (**endotelet**), samt orsaka obalans i kroppens blodstillningsförmåga (**hemostas**), vilket kan ge en ökad tendens till proppbildning (**koagulation**). Det är dock oklart ifall mikrovesikler i blodbanan är drivande i utvecklingen av småkärlssjuka vid typ 1 diabetes. Det finns även praktiska svårigheter med att analysera MVs och vi behöver förstå hur olika preanalytiska variabler kan påverka mätningar av MVs med flödescytometri. **Hudens mikrocirkulation** är en lättillgänglig modell för att studera mikrocirkulationen. Kopplingen mellan hudens mikrocirkulation och småkärlssjuka av olika svårighetsgrad vid typ 1 diabetes är dock ännu inte fastställd.

Syftet med den här avhandlingen var att studera relationen mellan mikrovesikler, mätningar av hudens mikrocirkulation och småkärlssjuka vid typ 1 diabetes. Vi försökte hitta möjliga biomarkörer som skulle kunna hjälpa kliniker att upptäcka mikroangiopati i ett tidigare skede.

Delarbete I och II studerade 236 patienter med och utan klinisk mikroangiopati samt 100 friska matchade kontroller. Patienter med typ 1 diabetes hade förhöjda nivåer MVs, där framförallt MVs från endotelceller var många gånger högre än hos friska. Vi såg en ökning av MVs som uttrycker HMGB1, ett protein som kan orsaka skador på endotelet. Det fanns dock inga tydliga skillnader i MV nivåer mellan patienter med och utan mikroangiopati. Plasma laktadherin, ett protein som hjälper till att bryta ner celler i blodbanan, var signifikant högre hos patienter med mikroangiopati än patienter utan. I **delarbete III** visade vi att singel-centrifugering innan frysning av plasmaprover, följt av re-centrifugering av tinade prover innan flödescytometri, gav lägre MV nivåer men samtidigt minskad mängd kvarvarande cellfragment jämfört med dubbelcentrifugering innan frysning, vilket minskar risken för falsk positiva fynd. I **delarbete IV** undersökte vi hudens mikrocirkulation hos 61 patienter med typ 1 diabetes och 31 kontroller. Störningar i hudens mikrocirkulation korrelerade till graden av mikroangiopati.

Sammantaget visade denna avhandling att patienter med typ 1 diabetes har förhöjda nivåer mikrovesikler, men utan relation till småkärlssjuka. Den kraftiga ökningen av endotel MVs och MVs som uttrycker HMGB1 kan innebära en ökad risk för framtida hjärt-kärlsjukdom. Lactadherin identifierades som en möjlig biomarkör för småkärlssjuka. Vårt enkla centrifugprotokoll skulle kunna tillämpas i kliniska studier för att studera MVs som biomarkörer vid en rad olika inflammatoriska sjukdomstillstånd. Störningar i hudens mikrocirkulation tycks spegla graden av klinisk mikroangiopati vid typ 1 diabetes.

POPULAR SCIENCE SUMMARY OF THE THESIS

Type 1 diabetes is a life-long autoimmune disease which causes the pancreas to lose its ability to control blood sugar. Despite treatment with insulin, patients frequently develop small vessel disease (**microangiopathy**), which may damage the kidneys (**nephropathy**), eyes (**retinopathy**) and nerves (**neuropathy**). Many develop decreased kidney function, vision loss, or chronic pain. Microangiopathy also contributes to patients with type 1 diabetes having a dramatically increased risk of heart disease.

Microvesicles (MVs) are small vessels released by all cells in the circulation, which act as key messengers to help cells communicate. MVs can promote inflammation, damage the inner lining of the blood vessels (**endothelium**) and cause disturbances in **hemostasis**, increasing the tendency of the blood to clot (**coagulation**). However, little is known about the role of plasma MVs as drivers of small vessel disease in type 1 diabetes. There are also practical difficulties with measuring MVs, and we need to understand how different pre-analytical steps affect MV analysis using flow cytometry. **Skin microcirculation** can be used as an accessible bedside model to study the microcirculation. However, the link between skin microcirculation and microangiopathy of different severity in type 1 diabetes is not yet clear.

The aim of this thesis was to study the relationship between plasma MVs, measurements of skin microcirculation and clinical microangiopathy in type 1 diabetes. We sought to identify potential biomarkers that might help clinicians identify microangiopathy at an earlier stage.

For **Papers I and II**, we compared 236 patients with type 1 diabetes, with and without microangiopathy, and 100 matched healthy controls. We found increased MVs amongst patients, and endothelial MVs were many times higher than in controls. Patients also had elevated MVs expressing HMGB1, an inflammatory protein that can damage the vessel wall. We found no clear differences in MVs between patients with and without microangiopathy. Lactadherin, a protein involved in the breakdown of cells in the blood, was significantly higher in patients with microangiopathy than in patients without. In **Paper III**, we showed that single centrifugation prior to freezing, followed by re-centrifugation of thawed samples prior to flow cytometry, resulted in decreased MV levels but also lower remaining cell fragments compared to a protocol involving double-centrifugation, which decreases the risk of false positive findings. In **Paper IV,** we measured skin microcirculation in 61 patients with type 1 diabetes and 31 healthy controls and showed that disturbances in skin microcirculation correlated with the severity of clinical microangiopathy.

In summary, this thesis found that patients with type 1 diabetes have elevated plasma microvesicles, but with no clear link to microangiopathy. Increased endothelial MVs and MVs expressing HMGB1 may increase the risk of future heart disease. Lactadherin is a potential new biomarker of microangiopathy in type 1 diabetes. Our simple centrifugation protocol could be used in clinical studies of MVs as potential biomarkers in different inflammatory conditions. Disturbances in skin microcirculation were shown to relate to the degree of clinical microangiopathy in type 1 diabetes.

ABSTRACT

Background: Type 1 diabetes is a proinflammatory and prothrombotic disease associated with a highly elevated risk of microvascular complications, including nephropathy, retinopathy, and neuropathy, as well as premature cardiovascular disease.

Aim: this thesis sought to study plasma microvesicles (MVs) and skin microcirculation in relation to clinical microangiopathy in type 1 diabetes, to try to identify potential clinical biomarkers. In a separate laboratory investigation, we explored how different centrifugation protocols affect flow cytometry measurement of MVs.

Methods: In **Papers I and II** we measured plasma MVs of different cellular origins and their expression of procoagulant phosphatidylserine (PS) and proinflammatory HMGB1 in 236 well-characterized patients and 100 matched healthy controls using flow cytometry. Plasma lactadherin was analyzed using ELISA. In **Paper III** we compared the MISEV 2018 centrifugation protocol to different single centrifugation protocols in terms of effect on MV levels and remaining cell fragments. In **Paper IV** we investigated skin microvascular reactivity in 61 patients with type 1 diabetes with and without microangiopathy and 31 healthy controls.

Results: In **Paper I** we found significantly higher levels of total plasma MVs, PS+ and PS- MVs in patients compared to controls, but with no correlation to clinical microangiopathy. Plasma lactadherin was increased in patients versus controls, and patients with microangiopathy had significantly higher levels than patients without. In **Paper II,** we found that subpopulations of both endothelial and platelet derived MVs were significantly increased in patients compared to controls. We also showed a significant increase in HMGB1+ MVs in type 1 diabetes. In **Paper III,** we demonstrated that a single round of centrifugation prior to freezing, followed by re-centrifugation after thawing, resulted in lower MV levels but also lower cell fragmentation than the MISEV 2018 protocol, decreasing the likelihood of false positive events. In **Paper IV**, we found that impairment in skin microvascular reactivity in patients with type 1 diabetes related to their degree of clinical microangiopathy.

Conclusions: Type 1 diabetes is associated with significantly increased MV levels, but with no relationship to clinical microangiopathy. The striking elevation of endothelial MV levels in type 1 diabetes points to pervasive endothelial dysfunction, whereas evidence of platelet hyperactivity was less pronounced. Elevated HMGB1+ MVs and plasma lactadherin in patients with type 1 diabetes could have implications for future vascular complications. A standardized centrifugation protocol using a single round of centrifugation prior to freezing, followed by re-centrifugation prior to flow cytometry analysis, was found to be a pragmatic approach for measuring MVs in larger clinical studies. Skin microvascular reactivity correlated with the severity of clinical microangiopathy in patients, suggesting that type 1 diabetes seems to affect the microvasculature in the whole body. Our findings support the use of skin microcirculation as a clinically relevant bedside model for the study of microangiopathy.

LIST OF SCIENTIFIC PAPERS

I. **Bergen, K.**, Mobarrez, F., Jörneskog, G., Wallén, H., & Tehrani, S. (2018). Phosphatidylserine expressing microvesicles in relation to microvascular complications in type 1 diabetes. Thrombosis Research, 172, 158-164.

II. **Bergen, K.**, Mobarrez, F., Jörneskog, G., Wallén, H., & Tehrani, S. (2020). High levels of endothelial and platelet microvesicles in patients with type 1 diabetes irrespective of microvascular complications. Thrombosis Research, 196, 78-86.

III. **Bergen, K.***, Aguilera Gatica, K.*, Tehrani, S., Wallén, H., Mobarrez, F., Flow cytometric analysis of extracellular vesicles and the influence of preanalytical handling. *co-authors with equal contribution Manuscript form.

IV. Tehrani, S., **Bergen, K.**, Azizi, L., & Jörneskog, G. (2020). Skin microvascular reactivity correlates to clinical microangiopathy in type 1 diabetes: A pilot study. Diabetes & Vascular Disease Research, 17, 1-9.

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

Bergen, K., Brismar, K., & Tehrani, S. (2016). High-dose atorvastatin is associated with lower IGF-1 levels in patients with type 1 diabetes. Growth Hormone and IGF Research, 29, 78-82.

CONTENTS

LIST OF ABBREVIATIONS

ACE-i	Angiotensin converting enzyme inhibitors
ACh	Acetylcholine
ACR	Albumin-creatinine ratio
AGEs	Advanced glycation end-products
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ARBs	Angiotensin II receptor blockers
AU	Arbitrary units
BMI	Body mass index
CAT	Calibrated, automated thrombogram
CBV	Capillary blood cell velocity
C.I.	Confidence interval
CVD	Cardiovascular disease
CTI	Corn trypsin inhibitor
CV	Coefficient of variation
ECs	Endothelial cells
eGFR	Estimated glomerular filtration rate
EMVs	Endothelial microvesicles
eNOS	Endothelial nitric oxide synthase
ETP	Endogenous thrombin potential
EVs	Extracellular vesicles
FSC	Forward scatter channel
HbA_{1C}	Glycated hemoglobin
HDL	High-density lipoprotein
HMGB1	High-mobility group binding protein 1
hsCRP	Highly sensitive C-reactive protein
ICAM-1	Intercellular adhesion molecule 1
IL-1	Interleukin 1
IL-6	Interleukin 6
IQR	Inter-quartile range

LDF	Laser Doppler fluxmetry
LDL	Low-density lipoprotein
NETs	Neutrophil extracellular traps
MFI	Mean fluorescence intensity
mRNA	Messenger ribonucleic acid
miRNA	Micro ribonucleic acid
MVs	Microvesicles
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NPP	normal pooled plasma
NPDR	Non-proliferative diabetic retinopathy
PBS	Phosphate-buffered saline
PMVs	Platelet microvesicles
PDR	Proliferative diabetic retinopathy
PORH	Post-occlusive reactive hyperemia
PPP	Platelet poor plasma
PS	Phosphatidylserine
RAGE	Receptor for advanced glycation end-products
RI	Refractive index
RT	Room temperature
SSC	Side scatter channel
SNP	Sodium nitroprusside
SD	Standard deviation
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TF	Tissue factor
TGFβ	Transforming growth factor beta
TNFα	Tissue necrosis factor alpha
VCAM-1	Vascular cell adhesion molecule 1

1 INTRODUCTION

1.1 TYPE 1 DIABETES

Type 1 diabetes mellitus (T1DM) is one of the most common chronic diseases affecting children and young adults worldwide, accounting for about 5-10% of all diabetes [1]. It is an immune-mediated disease characterized by chronic hyperglycemia resulting from insulin deficiency caused by the loss of the β-cells in the pancreas. For unknown reasons, the incidence rate of T1DM has increased at a rate of 3-4% annually since the 1950s [2], and there are currently over 1 million children under age 20 worldwide living with T1DM [3]. For unclear reasons, there is considerable regional variation in disease incidence and prevalence, with the Scandinavian countries being the most highly affected, experiencing incidence rates of 30-60/100 000 children per year [3].

T1DM is generally believed to be caused by a combination of genetic susceptibility and some unknown environmental trigger, which together leads to the initiation of an immune response against the pancreatic β-cells [2, 4, 5]. This autoimmune response involves a complex series of interactions between antigen presenting cells such as dendritic cells and B-cells, as well as CD4+ and CD8+ T-cells, leading to the activation of autoantigen-specific T-cells and a cascade of immune events ultimately resulting in the destruction of the β-cells in the pancreas [4, 6].

Even with modern-day treatment of hyperglycemia and concomitant risk factors such as hypertension and dyslipidemia, patients with T1DM experience a highly elevated risk of developing vascular damage to the smallest blood vessels in the body, referred to as microangiopathy or microvascular disease. Since patients often develop the disease in early childhood, they may be afflicted by microvascular complications as early as in their teens or early twenties, after a diabetes duration of 10-20 years [7-9]. The most common clinical manifestations involve the retina, glomeruli, and peripheral nerves [4]. Diabetic retinopathy is the leading cause of blindness in working-age adults [10] and diabetic nephropathy is the leading cause of chronic kidney disease and end-stage renal disease worldwide [11], although most studies have looked at both type 1 diabetes and type 2 diabetes in combination.

1.2 TYPE 1 DIABETES AS A PROTHROMBOTIC STATE

As a result of improvements in medical management, both the risk of cardiovascular disease (CVD) as well as the overall life expectancy of patients with type 1 diabetes have improved considerably in the past decades [12-14]. Nevertheless, patients with type 1 diabetes remain disproportionately affected by vascular disease, with CVD being the main cause of excess mortality [14]. In 2014, the American Heart Association and American Diabetes Association released a joint scientific statement identifying the urgent medical need to improve our understanding of the mechanisms driving the increased risk of heart disease in patients with

T1DM, which has a distinct pathophysiology and typically much younger age of onset than type 2 diabetes mellitus (T2DM) [15]. Livingstone et al. showed that patients with T1DM at age 20 have an estimated life expectancy that is approximately 11 years shorter for men and 13 years shorter for women, compared to their nondiabetic counterparts, mostly accounted for by the increased risk of dying of ischemic heart disease [16]. A recently published study looking at Swedish patients with type 1 diabetes between years 2017-2019 found rates of myocardial infarction, heart failure and stroke to be 3.4-5.0 times higher in patients with type 1 diabetes compared to the general population [14]. There is also evidence suggesting that cardiovascular outcomes are worse for patients with T1DM compared to T2DM [17].

The Diabetes Control and Complications Trial and the long-term follow up of the Epidemiology of Diabetes Interventions and Complications Trial conclusively demonstrated that both microvascular and macrovascular disease in type 1 diabetes are clearly linked to metabolic control, and that intensive insulin therapy aimed at decreasing blood glucose levels to more physiological levels can decrease long-term vascular complications [18, 19]. However, even with stringent metabolic control, current medical management is unable to completely ameliorate the excess risk of vascular disease experienced by patients with the disease. Lind and colleagues demonstrated that patients who reach target levels for glycated hemoglobin (HbA_{1C}) levels of <52 mmol/mol still have twice the all-cause mortality and CVD mortality as matched controls [20].

In addition, patients with T1DM have more frequent episodes of hypoglycemia and higher glucose variability than patients with T2DM [21]. HbA_{1C} variability has been shown to be an independent predictor of microvascular complications in type 1 diabetes [22]. A single serious hypoglycemia episode is associated with an increased risk of future CVD and all-cause mortality [23], and repeated hypoglycemia episodes have been linked to subclinical atherosclerosis [24]. This is likely related to hypoglycemia-induced activation of the sympathetic nervous system, leading to activation of procoagulant and proinflammatory pathways, platelet aggregation and disturbances in endothelial dysfunction [25]. Insulin resistance may also contribute to increased glucose variability and a more prothrombotic vascular profile in some patients with T1DM [26, 27].

Apart from problems with regulating blood sugar, other traditional cardiovascular risk factors, including hypertension, obesity, dyslipidemia, and smoking, also contribute to cardiovascular risk in T1DM [25, 28-30]. Importantly, Rawshani et al. found that even T1DM patients who meet all their treatment goals with regards to HbA_{1C}, blood pressure, albuminuria, smoking, and blood lipids, still experience an excess risk of acute myocardial infarction and heart failure [31]. There is thus an urgent need to better understand the prothrombotic nature of type 1 diabetes, in order to be able to improve patient care in the long-term.

1.2.1 The blurring distinction between micro- and macrovascular disease

Research in recent years has broadened our understanding of the complexity of the pathophysiology of vascular disease in T1DM and called into question the tradition of classifying disease manifestations as being either macrovascular or microvascular in nature. Microangiopathy should rather be viewed as a general process occurring in the whole body, which plays an essential role in the pathophysiology of organ damage that has otherwise been viewed as being caused predominantly by damage to the larger vessels, such as heart disease and stroke (Figure 1) [32, 33]. Cerebral microvascular disease has also been shown to contribute to the development of cognitive dysfunction and depression in T1DM [34, 35]. In addition, there is a strong correlation between microvascular disease and the development of heart failure in T1DM [36].

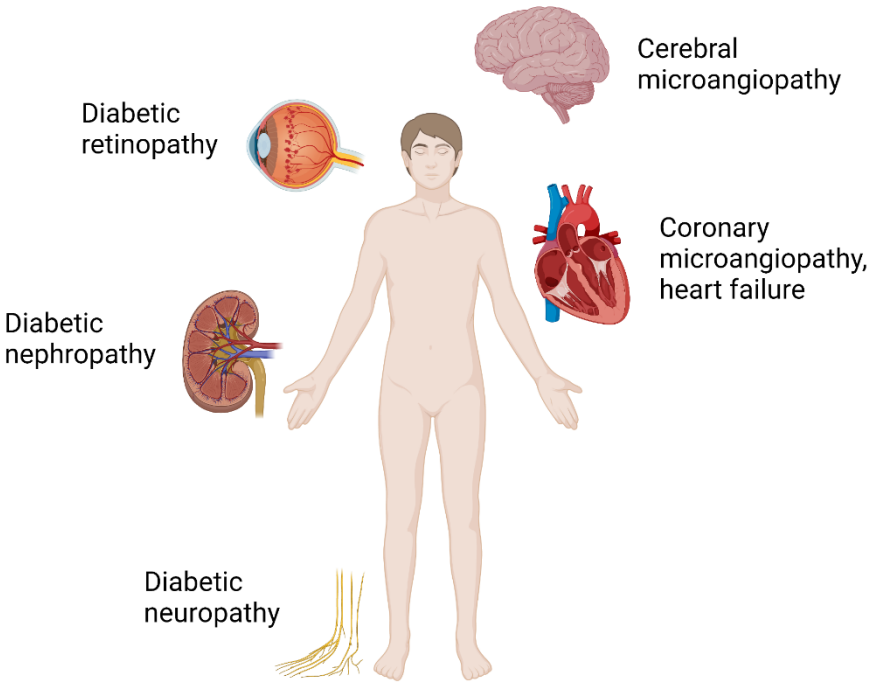


Figure 1. Manifestations of microangiopathy in type 1 diabetes.
Typically recognized microvascular organ manifestations are shown on the left, whereas organ manifestations for which the contribution of microvascular disease is sometimes overlooked are shown on the right. Created with Biorender.com.

Even after controlling for known cardiovascular risk factors, diabetic nephropathy has been demonstrated to be a strong independent risk factor for developing ischemic heart disease in T1DM [28, 37], and diabetic nephropathy has a strong negative impact on excess mortality [14]. Inflammation, endothelial dysfunction, and hypercoagulability have been proposed as shared pathophysiological mechanisms that underlie both micro- and macrovascular disease [38, 39]. A recent meta-analysis found that T1DM is associated with premature development of endothelial dysfunction in both small and large blood vessels [40].

1.2.2 Sex differences in vascular disease in type 1 diabetes

For unclear reasons, women with type 1 diabetes lose the natural protection that healthy pre-menopausal women have against CVD [41]. Numerous studies have found that women with type 1 diabetes experience a much greater relative risk increase compared to men with type 1 diabetes, even after adjusting for known CVD risk factors [16, 42-45]. Colhoun et al. showed that type 1 diabetes abolished the sex differences in coronary artery calcification present in healthy men and women, but this could only partially be explained by known risk factors of atherosclerosis [46]. It is possible that this disproportionate increase in cardiovascular risk could at least partially be explained by a greater disturbance in hemostatic function in women with type 1 diabetes, as Tehrani et al. found that young women with type 1 diabetes have a tighter fibrin network with longer lysis time than corresponding men [47].

1.2.3 Protective factors and the need for new biomarkers

Remarkably, even in the setting of chronic hyperglycemia, a significant subset of patients with type 1 diabetes will live for many years without ever developing microvascular complications [48], as demonstrated by the Joslin Diabetes Center 50-Year Medalist Study [49]. Also, studies have shown that a subset of patients with microalbuminuria regress to normoalbuminuria independently of medical treatment with angiotensin converting enzyme inhibitors (ACE-i) [50, 51]. These findings suggest that there are individual protective factors that guard certain patients from disease progression. Recently, results from the PROLONG and DIALONG trials, two large cross-sectional Scandinavian cohorts of patients with type 1 diabetes who have lived with the disease for over 30 years, showed that certain polymorphisms in genes coding for the interleukin 2 receptor as well as liver synthesis of specific nucleotides involved in promoting insulin sensitivity were associated with the risk of developing microvascular complications [52, 53]. However, our understanding of the pathophysiology of microvascular complications and what factors predispose certain patients to being more affected than others remains incomplete.

Another important consideration is that our current ability to detect the presence of microvascular complications in the clinic is limited, since pathological changes to the microvasculature can be present for years before overt clinical signs develop. For instance, the International Diabetic Nephropathy Study looking at consecutive renal biopsies in 243 patients with T1DM showed that increased glomerular basement membrane thickness and mesangial matrix expansion were typically the earliest indications of diabetic nephropathy, often present for several years before any significant leakage of albumin in the urine could be detected [54].

There is thus an urgent need not only to improve our understanding of the pathophysiological mechanisms contributing to vascular disease in T1DM [25], but also to identify biomarkers to be able to detect and predict vascular complications in patients with T1DM at a much earlier stage. A biomarker is any characteristic such as a molecule or physiological measurement that can be used as an indicator of an underlying physiological or pathological biological process [55].

1.3 CLINICAL ASSESSMENT AND CLASSIFICATION OF MICROANGIOPATHY

Estimates of the prevalence of microvascular complications in type 1 diabetes vary considerably by region and age-group. Data from Australia and Sweden suggest that about one in five adolescents with type 1 diabetes is affected by diabetic retinopathy within a few years of disease onset [56, 57], and after 30 years fewer than 10% of patients with type 1 diabetes are completely free from retinopathy [58]. A study looking at the prevalence of renal complications in four national cohorts from Sweden, Austria, Germany and the US between 2016-2018 found that of patients with type 1 diabetes and disease duration less than 20 years, around 10% had albuminuria and 17% had decreased kidney function, compared to about 1/3 of patients with albuminuria and 25% with decreased kidney function after more than 40 years [59].

1.3.1 Diabetic nephropathy

The gold standard for identification of diabetic nephropathy is histopathological assessment of a kidney biopsy sample. In practice, however, most patients are diagnosed based on clinical assessment in combination with laboratory findings, including persistent albuminuria that is confirmed with at least 2 urine samples 3-6 months apart and/or a decline in estimated glomerular filtration rate (eGFR), where other causes of kidney disease have been deemed unlikely. Albuminuria is categorized as normal to mildly increased if a person has an urine albumin-creatinine ratio (ACR) < 3 mg/mmol, moderately increased if ACR is 3-30 mg/mmol (microalbuminuria) and severely increased if ACR is > 30 mg/mmol (macroalbuminuria) [60].

1.3.2 Diabetic retinopathy

Diabetic retinopathy can be diagnosed and graded through the use of stereoscopic fundus photographs, with the Early Treatment Diabetic Retinopathy Study classification system from 1991 [61] still considered to be a gold standard by many. In clinical practice, diabetic retinopathy is typically divided into either non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR), depending on the presence of abnormal new blood vessels. NPDR can be further classified as mild, moderate or severe and PDR can be divided into high-risk or non-high-risk [62]. Early signs of clinically detectable NPDR (simplex or background retinopathy) typically involve the development of retinal microaneurysms and blot hemorrhages. As the disease progresses, hard exudates may appear due to lipoproteins leaking into the retina through a defective microvascular barrier, as well as soft exudates or cotton wool spots, the result of nerve fiber infarctions in the retina. Continued retinal ischemia results in intraretinal microvascular abnormalities, such as dilated microvessels, as well as venous beading. PDR is identified by the appearance of fragile new retinal blood vessels that may bleed into the vitreous space, as well as fibrosis near the optic disk or venules, which can result in pulling of the macula, ultimately causing vision loss [32].

1.3.3 Diabetic neuropathy

Diabetic neuropathy in the form of distal symmetric sensory polyneuropathy can be screened for in the clinic through a relatively simple neurological exam, although various severity scales also exist. Loss of large nerve fibers leads to decreased proprioception, vibratory sensation (assessed with a 128 Hz tuning fork) and peripheral reflexes in the legs (assessed by examining achilles and patellar reflexes), whereas damage to small nerve fibers leads to impaired sensation of pain, temperature and light touch (tested with monofilament) [63]. Clinical signs of autonomic neuropathy involving the sympathetic and parasympathetic nervous system include delayed gastric emptying, so called gastroparesis, which can cause abdominal discomfort, nausea, and vomiting and contribute to malnutrition in patients with T1DM. Other signs of autonomic neuropathy include orthostatic hypotension and erectile dysfunction [63].

1.4 PATHOPHYSIOLOGY OF MICROANGIOPATHY

The pathophysiology of microangiopathy remains incompletely understood but involves a complex series of alterations to the microcirculation, including degraded glycocalyx, basal membrane thickening, endothelial dysfunction, disturbed microvascular autoregulation, vascular inflammation, hypercoagulability, and platelet hyperactivity, as reviewed in more detail elsewhere [4, 32, 39, 40, 64]. Apoptosis of support cells and progenitor cells is increased, resulting in secondary damage and a diminished regenerative capacity in the vasculature and other organs [64]. The precise mechanism of damage is specific to each organ, but there are also common pathways that lead to progressive cell damage, eventually manifesting in the form of clinically detectable microangiopathy.

1.4.1 Inflammation

Type 1 diabetes induces a multitude of proinflammatory disturbances to the intravascular milieu. Some of the most important mechanisms are reviewed below (Figure 2).

1.4.1.1 Reactive oxygen species

Hyperglycemia in T1DM not only directly damages the vascular endothelium, smooth muscle cells and surrounding support cells, but also indirectly leads to injury through overwhelming the oxidative phosphorylation capacity of the mitochondria. This leads to the production of excess reactive oxygen species (ROS), such as superoxide, which have potent cell-damaging and proinflammatory effects [32].

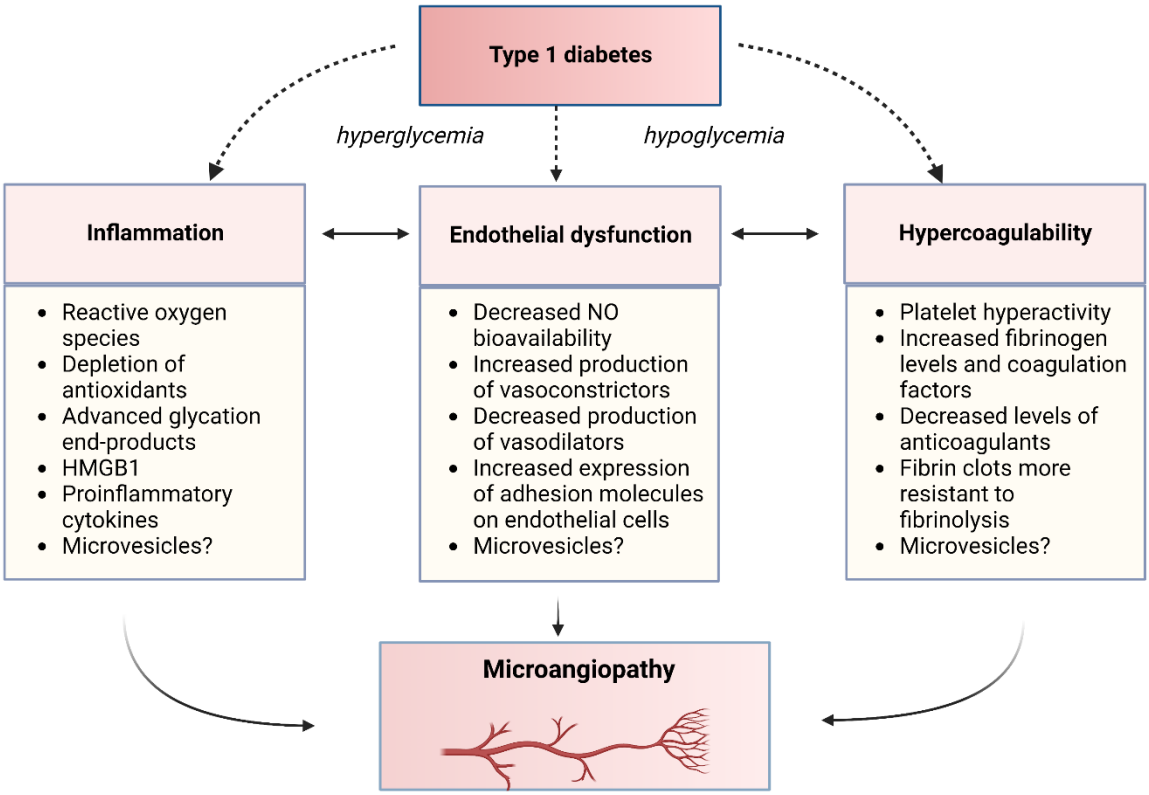


Figure 2. Simplified overview of the pathophysiology of microangiopathy in type 1 diabetes.

Hyperglycemia as well as hypoglycemia in type 1 diabetes trigger inflammation, endothelial ddysfunction, and hypercoagulability, ultimately leading to the development of microangiopathy. HMGB1, high-mobility group box protein 1. NO, nitric oxide. Created with Biorender.com.

1.4.1.2 Advanced glycation end-products

In the setting of chronic hyperglycemia, glucose undergoes covalent bonding to different proteins in the body, leading to the creation of advanced glycation end-products (AGEs), which can inhibit or alter their normal function. AGEs cross-linking to collagen can cause basement membrane thickening [65], which in turn impairs oxygen diffusion to the surrounding tissue cells and affects the permeability of the endothelial lining. In a cross-sectional study of 351 patients with type 1 diabetes and disease duration > 50 years, the presence of AGEs was highly correlated with the presence of microvascular complications [66]. AGEs can also interact with the membrane receptor for advanced glycation end-products (RAGE), which through a series of intracellular signaling events involving the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) leads to altered gene expression and the production of proinflammatory cytokines such as interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor α (TNF-α). These pathological changes in turn may act to promote endothelial dysfunction. For instance, TNFα causes upregulation of the adhesion molecule E-selectin, expressed by activated endothelial cells, and soluble forms of E-selectin have been found to be increased in T1DM and correlate with the progression of retinopathy as well as nephropathy

[67, 68]. NF-κB also increases the production of intercellular adhesion molecule 1 (ICAM-1), expressed on leukocytes and endothelial cells. T1DM is associated with elevated levels of soluble ICAM-1, with a correlation to clinical signs of microangiopathy and impaired skin microvascular reactivity [69].

1.4.1.3 HMGB1

High-mobility group box protein 1 (HMGB1) is a chromatin-binding protein involved in regulating DNA transcription and in the maintenance of homeostasis. It can also be translocated from the cytosol to the extracellular surface either actively in immune cells such as monocytes, macrophages or dendritic cells, or passively upon apoptosis or necrosis of the cell [70]. Extracellular HMGB1 acts as an alarmin or damage-associated molecular pattern receptor, which upon sensing high glucose or other stressors initiates a cascade of downstream signaling events through interaction with RAGE, toll-like receptors, and the NF-κB signaling pathway, thus creating a type of sterile inflammation with upregulation of IL-1 and TNFα, ROS-formation and other proinflammatory mediators [71, 72]. HMGB1 causes upregulation of ICAM-1, vascular cell adhesion molecule 1 (VCAM-1) and E-selectin on endothelial cells (ECs), thus promoting increased endothelial-leukocyte crosstalk and contributing to the breakdown of the normal barrier function of the endothelium [73]. HMGB1 also plays an important role in platelet-neutrophil interactions, with HMGB1+ platelets being able to induce formation of neutrophil extracellular traps (NETs), thus contributing to thromboinflammation [74].

As reviewed in detail elsewhere, HMGB1 has increasingly been recognized as a key proinflammatory mediator in the development of both microvascular and macrovascular disease in diabetes [70, 71, 75-79], although the majority of studies have been undertaken either in-vitro, in animal models or in patients with type 2 diabetes. A mechanistic study in diabetic rats studied the effect of HMGB1 on the endothelial barrier in the retina and demonstrated that intravitreal injections of HMGB1 led to increased production of adhesion molecules such as ICAM-1, inflammatory mediators including RAGE and NF-κB and also increased retinal vascular permeability [80]. HMGB1 has been shown to stimulate tubulointerstitial inflammation in diabetic nephropathy in rats [78], as well as promote fibrosis in cultured renal tubular epithelial cells [81]. Blocking HMGB1 has been found to attenuate development of diabetic nephropathy in mice [82] and decrease podocyte apoptosis through inhibition of the effects of transforming growth factor beta (TGFβ) [83]. A small study recently reported that HMGB1 appears to be important in mediating ferroportosis, a newly discovered type of programmed cell death involved in inflammation, in mesangial cells in the kidney of diabetic patients [84].

1.4.2 Endothelial dysfunction

The endothelial cells lining blood vessels play a critical regulatory role in the maintenance of vascular integrity. The term endothelial dysfunction has traditionally been used to describe a disturbance in the ability of the endothelium to regulate vascular tone. A key feature of endothelial dysregulation is decreased levels of nitric oxide (NO), an essential vasodilator as well as key mediator of vascular homeostasis in the body. NO acts by diffusing freely into smooth muscle cells and platelets, where it activates guanylate cyclase, which in turn leads to the production of cyclic guanosine monophosphate, an inhibitor of platelet aggregation and promotor of vascular relaxation [85]. NO also has important antioxidant properties [86]. NO bioavailability is decreased in T1DM through several biochemical pathways. Excess glucose is shunted into non-glycolytic pathways such as the polyol pathway, leading to increased sorbitol production, which contributes to hyperosmolarity and oxidative stress [64]. The production of sorbitol also depletes NADPH stores, a necessary cofactor for the intracellular antioxidant glutathione [32] as well as endothelial nitric oxide synthase (eNOS), thereby contributing to diminished antioxidant capacity and decreased production of NO. In addition, hyperglycemia leads to activation of protein kinase C, a cytoplasmic enzyme that decreases eNOS synthesis, while simultaneously increasing production of vasoconstrictors, such as angiotensin II and endothelin-1 [85, 87]. Angiotensin II plays a particularly important role when it comes to development of diabetic nephropathy, both as a result of its vasoconstrictive effect on the glomerular microcirculation, and because of initiation of downstream signaling events contributing to fibrosis through TGFβ signaling [32, 88]. Endothelial dysfunction also involves a decrease in prostacyclin, an important vasodilator and platelet inhibitor, which again shifts the balance towards a more prothrombotic state.

Damage to the glycocalyx covering the luminal side of the endothelium is believed to be another important component of endothelial dysfunction, causing defective microvascular permeability [89, 90].

In recent years, the term endothelium dysfunction has started to be applied more broadly to encompass any maladaptive changes that alter the normal physiology of the endothelium and disturbs its many biological functions, including its important anti-thrombotic properties, role in cell adhesion, control of vessel wall inflammation, and regulation of platelet activity [91].

1.4.3 Hypercoagulability

In addition to the procoagulant effect of inflammation and endothelial dysfunction, T1DM is also associated with several other hemostatic derangements that shift the hemostatic balance in the vasculature towards a more prothrombotic state, as reviewed extensively elsewhere [92, 93]. Hemostatic alterations are detectable early following disease onset and appear to be linked to the development of microvascular complications in T1DM, with patients with clinical microangiopathy experiencing more pronounced hemostatic derangements compared to those without [47, 94]. Diabetes-related hemostatic disturbances include platelet hyperactivity [95],

increased levels of fibrinogen [94], elevated tissue factor procoagulant activity [96], abnormally high levels of several coagulation factors including FV, FVIIa, FVIII, FX and prothrombin, as well as higher thrombin generation in-vitro [97]. Patients with T1DM also have decreased levels of anticoagulant proteins such as protein C [93, 97] and form tighter fibrin clots that are more resistant to fibrinolysis [47]. The deficient levels of insulin in T1DM in itself predisposes to hyperactive platelets, since insulin normally acts to inhibit the adenosine diphosphate receptor P2Y12, which is involved in platelet response to procoagulant stimuli [93]. Some but not all of the hemostatic changes seem to be reversible by improved metabolic control [93]. Importantly, the results of several prospective studies suggest that biomarkers related to these hemostatic disturbances may be able to predict future onset of both microvascular complications as well as cardiovascular disease, independently of other traditional risk factors [85, 87, 92].

1.5 MICROVESICLES

1.5.1 What are MVs?

Extracellular vesicles (EVs) are nano-sized particles released by all cells in the circulation into the extracellular environment upon either apoptosis or activation in response to various physiological or pathological stimuli, including hyperglycemia, C-reactive protein, urea and shear stress, among others [98, 99]. EVs can act as biological vectors through expression of surface signaling molecules from their parental cells as well as through transfer of internal cargo including proteins, bioactive lipids, messenger ribonucleic acids (mRNA), and micro ribonucleic acids (miRNA) that can affect intracellular signaling and alter gene expression in recipient cells [100, 101].

EV is an umbrella term used to include both smaller exosomes, 30-100 nm in diameter, as well as larger microvesicles (MVs), 50-1000nm in diameter, the latter which are also frequently referred to as microparticles. As shown in Figure 3 below, exosomes are derived from the endocytic pathway where the cell plasma membrane invaginates inwards, forms lipid aggregates called mature multivesicular bodies and then fuses with the plasma membrane before being released, while MVs are formed by direct outward budding of the plasma membrane [102]. As the understanding of the complexity of isolating and identifying EVs has increased, it has become clear that the ability to accurately distinguish between these two populations in studies is not always possible, and the term EVs has therefore been proposed [103], although many researchers still choose to focus their work specifically on either smaller EVs i.e. exosomes, or larger EVs, i.e. MVs. The term EVs also encompasses apoptotic bodies released from apoptotic cells as well as a newly discovered fourth type of nanoparticle without a phospholipid membrane called exomeres, whose physiological function is not yet clear [104].

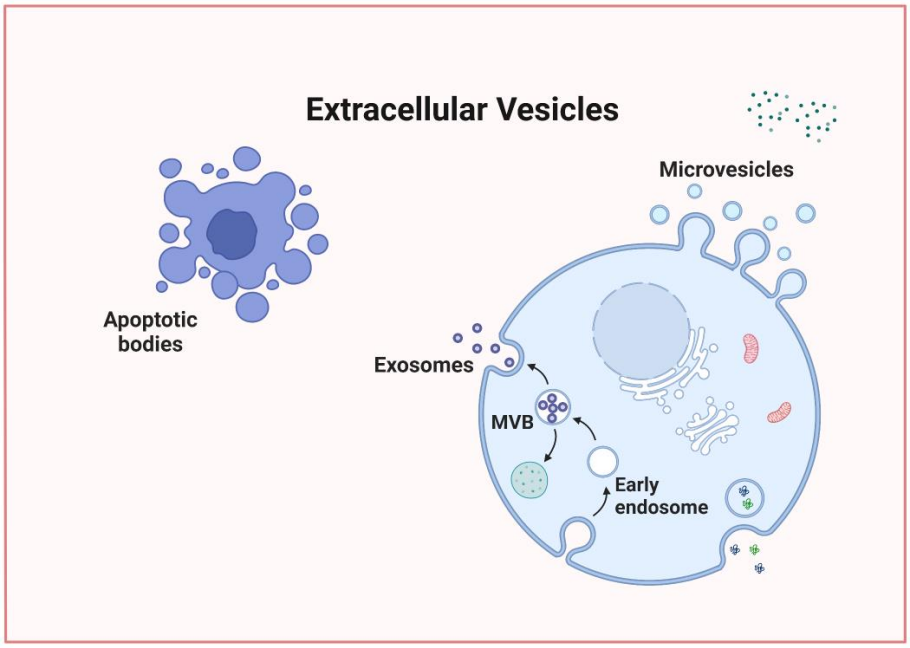


Figure 3. Formation of the three main types of extracellular vesicles.
Microvesicles are formed by direct outward budding of the phospholipid membrane. MVB, multi-vesicular bodies. Figure created with BioRender.com using a modified version of the "Extracellular Vesicle Separation by Density Gradient Ultracentrifugation" template.

Although the exact number of EVs in the circulation under physiological conditions remains a source of debate, there is consensus that the majority of EVs are released from megakaryocytes and platelets [105]. Endothelial EVs normally constitute only a small percentage of the total EV population in the circulation, but numbers increase under conditions associated with endothelial dysfunction, which can also affect their expression of inflammatory markers and cargo content [106].

1.5.1.1 Exposure of phosphatidylserine on the surface of EVs

In resting cells, the lipid bilayer is maintained in an asymmetric state through the action of the constitutively active adenosine triphosphate-dependent transporter flippase, governing inward transportation of phospholipids, as well as the much lower activity of floppase, governing outward transportation, together with scramblase, favoring non-specific bi-directional translocation. As such, the inward leaflet is primarily made up of phosphatidylserine (PS) and phosphatidylethanolamine, whereas the outer leaflet expresses sphingomyelin and phosphatidylcholine. Upon stimulation by an agonist, however, there is a calcium-dependent inhibition of flippase and concurrent upregulation of floppase and scramblase. This leads to a transient loss of asymmetry, exposure of PS on the outside of the lipid bilayer and concomitant membrane budding and shedding of EVs expressing PS [107]. Research in recent years, however, have also identified a significant subset of EVs that are PS-negative, but the nature of their release from their parental cell is not yet clear [108].

1.5.2 MVs as drivers of inflammation, endothelial dysfunction, and thrombosis

As reviewed in detail elsewhere, MVs are increasingly being recognized as key regulators of inflammation, endothelial function, coagulation, and thrombosis [109-112]. Importantly, under procoagulant conditions, MVs can express procoagulant PS on the outer leaflet, as well as the coagulation initiator tissue factor (TF). As shown in Figure 4 below, PS+ MVs can act as a catalytic surface to propagate coagulation by binding coagulation factors V [113] and VIII [114], which facilitates the formation of the tenase (factors VIIIa, IXa, X) and prothrombinase (factors Va, Xa and prothrombin) complexes and thus promotes the formation of a stable blood clot [115]. The degree to which TF exposure contributes significantly to MV promotion of coagulation in-vivo may vary in MVs of different cellular origin [116] and remains somewhat unclear, with some researchers suggesting that it is of limited importance [117]. These disparate findings may result from the inherent complexity of TF biology, where TF may exist in both an encrypted and an active state, as well as current deficiencies in the activity assays available to study TF [118, 119].

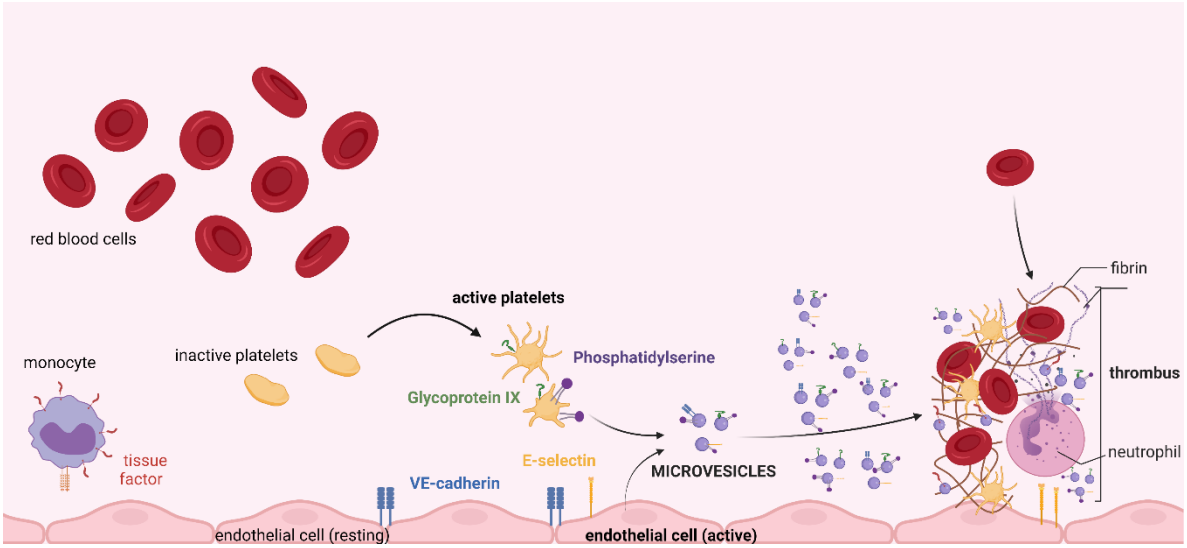


Figure 4. Contribution of circulating microvesicles to thrombus formation.

Under proinflammatory conditions, such as hyperglycemia, platelets and endothelial cells can become activated and release microvesicles (MVs) into the circulation. A subset of MVs may express phosphatidylserine on their surface, which can act as a catalytic surface for the formation of the tenase (factors VIIIa, IXa, X) and prothrombinase (factors Va, Xa and prothrombin) complexes in the coagulation cascade, helping to expediate thrombus formation. Figure created with BioRender.com using a modified version of the "Blood vessel and interstitium" template.

Apart from their role in furthering thrombosis, platelet MVs (PMVs) can release proinflammatory cytokines including IL-1, IL-6 and TNFα, induce activation and expression of adhesion molecules by ECs as well as increase monocyte recruitment and endothelial transmigration of leukocytes [112]. TNFα in turn promotes release of endothelial MVs (EMVs)

with increased expression of adhesion molecules such as ICAM-1 and VCAM-1, which leads to additional release of cytokines and enhanced EC-leukocyte interactions, creating a self-perpetuating proinflammatory cycle [112].

Hyperglycemia has been shown to increase formation of MVs and cause a shift towards a more prooxidative profile in cultured ECs [120]. Huang and colleagues recently demonstrated that PMVs are deposited in podocytes and incite inflammation that contributes to diabetic nephropathy [121]. Type 2 diabetes is associated with higher levels of both PMVs and EMVs as well as a shift towards more procoagulant MVs that can promote thrombus formation in-vitro and mediate endothelial dysfunction [122]. Several studies have found a correlation between higher MV levels in T2DM and microvascular complications and MVs have also been shown to act as independent predictors of cardiovascular disease, as reviewed extensively elsewhere [98, 102, 105, 108, 109, 111, 122-130].

1.5.3 The role of MVs in microangiopathy in type 1 diabetes

Numerous in-vitro experiments in the setting of hyperglycemia as well as animal models of type 1 diabetes support the role of MVs as important drivers in the development of microvascular complications in T1DM [120, 131-134]. However, the number of studies on humans is limited. Sabatier et al. was the first to demonstrate that patients with T1DM have a distinct MV profile compared to patients with T2DM [135]. They studied PMVs, EMVs and total PS+ MVs in 24 patients with T1DM (mean age 34, mean diabetes duration 12 years) and 52 patients with T2DM (mean age 57, mean disease duration 10 years). They found that EMV and PMV levels were significantly elevated in T1DM compared to age-matched controls, whereas only total MV numbers were elevated in T2DM. The procoagulant activity of PS+ MVs isolated from T1DM was increased, which was not the case for T2DM, and the total MV numbers were also higher in T1DM compared to T2DM. EMV levels were positively correlated with albuminuria in T1DM and patients with microvascular complications had higher EMV levels than those without, although the sample size was too small to draw any firm conclusions [135].

Salem et al. studied PMV levels in 80 children and adolescents with T1DM including 40 with and 40 without microvascular complications as well as 40 healthy controls [136]. They found that patients with T1DM had significantly higher levels of PMVs compared to healthy controls, and patients with microalbuminuria had significantly higher PMVs than those with normoalbuminuria, with a trend towards higher PMV levels in patients with neuropathy and retinopathy. PMV levels correlated positively with urine albumin-creatinine ratio, HbA_{1C}, highly sensitive C-reactive protein (hsCRP) and carotid intima thickness. The authors concluded that that PMVs were a significant independent risk factor for microvascular complications as well as a possible link between micro- and macroangiopathy [136].

These studies suggest that MVs are increased in patients with T1DM compared to healthy controls, with some evidence of a correlation to microangiopathy. However, larger studies as

well as studies comparing MV levels in adult patients with different severity of microangiopathy are needed in order to validate the use of MVs as biomarkers for early detection of microvascular complications in T1DM.

1.5.4 Methods to study MVs and their prothrombotic potential

1.5.4.1 Flow cytometry

Flow cytometry is the main method used to detect EVs in larger studies. An illustration of the basic setup for flow cytometry analysis of MVs is depicted in Figure 5 below.

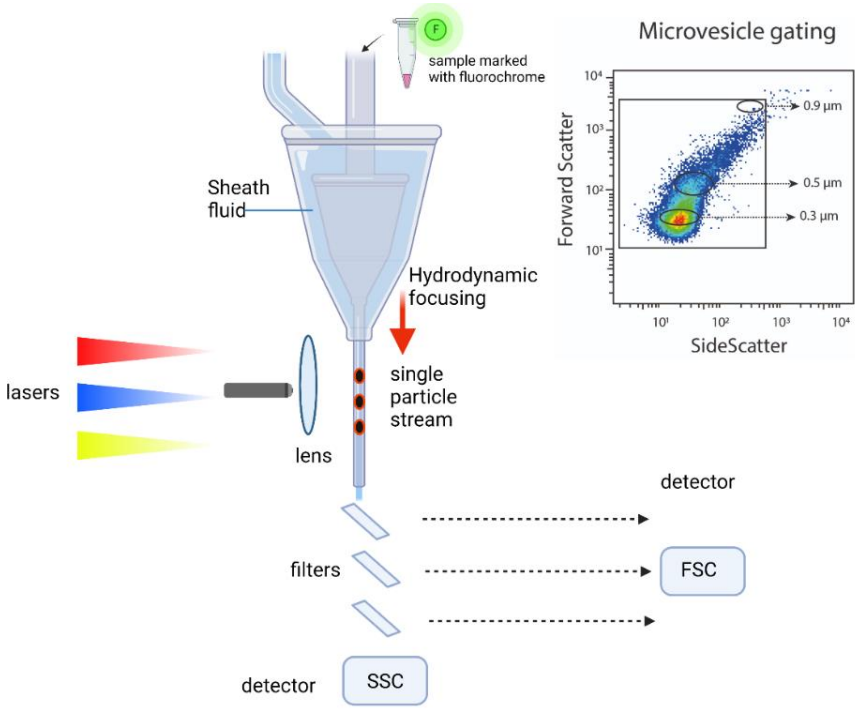


Figure 5. Illustration of flow cytometry analysis of microvesicles.
FSC, forward scatter. SSC, side scatter. Created with BioRender.com

In brief, cells or particles in a suspension are injected into a thin tube with a surrounding laminar flow of sheath fluid that through so called hydrodynamic focusing arranges the cells into a single-cell stream for analysis. The particles are typically pre-incubated with fluorescence-labeled antibodies (fluorochromes or fluorophores) that bind an antigen of interest on the cell. Upon exiting the tube, a laser beam excites the particle, after which a series of optical filters or dichroic mirrors with specific transmission spectra direct the light emission from the particle to a photodetector. The photodetector, usually a photomultiplier tube, transforms the emitted light photons to photoelectrons, thereby generating a small current whose voltage is proportional to the number of photons. The voltage is amplified to allow for better detection

and the analog signal is digitized and further processed for data analysis by a computer. Light that is scattered in the forward direction (usually 20°) is collected by the forward scatter channel (FSC) and is dependent on the size of the particle. Light that is scattered at a perpendicular angle is detected by the side scatter channel (SSC) and is determined by the granularity of the particle. The FSC and SSC signature will as such be unique for each particle. Fluorochromes in combination with several different lasers and detectors (fluorescence channels) for capturing different wavelengths will in turn allow for detection of multiple antigens on the same cell, although the maximum number of markers that can be detected simultaneously is limited due to the emission spectra from fluorescent tags overlapping [137].

1.5.4.2 The calibrated, automated thrombogram

As illustrated in Figure 6 below, assessment of the overall hemostatic potential of a biological sample or isolated particle can be studied through use of the calibrated, automated thrombogram (CAT) [138]. CAT is an ex-vivo thrombin generation assay that quantifies the generation of thrombin in plasma by the ability of thrombin to selectively cleave a fluorogenic substrate, causing the emission of a signal that can be recorded as fluorescence intensity, which in turn will be proportional to the amount of thrombin generated. The fluorescence signal is calibrated by simultaneously comparing the fluorescence intensity from the test sample with that emitted from a non-clotting plasma sample with a known thrombin concentration. The method can be used either with or without the addition of coagulation activators in the form of calcium, phospholipids, and TF. The thrombin generation curve is then analyzed by looking at several different parameters, including the lag-time, time to peak, peak height, and the endogenous thrombin potential (ETP), determined by the area under the curve over 60 minutes and corresponding to total thrombin formation [139]. These parameters will give an indication of the coagulation state of the sample.

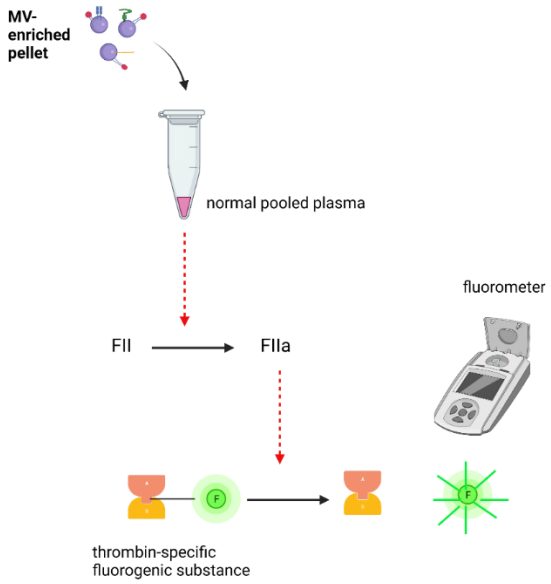


Figure 6. Setup for measuring microvesicle-induced thrombin generation in-vitro using a modified version of the calibrated, automated thrombogram.

FII, coagulation factor II. F is used to denote fluorophore. Created with BioRender.com

1.6 SKIN MICROCIRCULATION

The body´s microcirculation constitutes about 99% of the blood vessels in the body and consists of small arteries, arterioles, capillaries, and post-capillary venules with a diameter less than 150 µm in diameter [140]. The microcirculation is responsible for the transfer of oxygen, nutrients, and solutes, and dynamically regulates blood flow to adapt to shifts in the metabolic demands of the surrounding tissue. The skin microvasculature serves an important function in thermal regulation [141]. The skin microcirculation consists of two parallel plexa, one superficial and one deep, connected by arteriovenous anastomoses [140]. The skin blood flow is regulated through a combination of humoral, endothelial, and neural mechanisms, where sympathetic control can exert both a noradrenergic vasoconstrictor as well as an active vasodilator effect. Arteriovenous anastomoses and precapillary smooth muscle cells can regulate blood flow and thus maintain normal body temperature. At rest, vasoconstrictors typically maintain low skin perfusion. When body heat increases or during exercise, the skin perfusion is increased through both sympathetic vasodilating signals as well as the action of vasodilators released from endothelial cells [142].

Assessment of the cutaneous microvasculature has been proposed to be an easily accessible method for non-invasive studies of microvascular dysfunction in-vivo [143-145], with the important caveat that microcirculation in different parts of the body can also vary in their regulation according to their specific physiological function [146].

1.6.1 Skin microvascular dysfunction in type 1 diabetes

The pathophysiological processes underlying microangiopathy in T1DM not only affect the inner organs of the body, but also cause microvascular dysfunction in the skin. Diabetes is associated with abnormalities in skin microvascular reactivity including a diminished capacity for autoregulation of blood flow as well as a decreased vasodilatory reserve following provocation [147].

Several previous studies using laser Doppler imaging coupled with iontophoresis, a method for delivery of vasodilatory stimuli through the skin, have demonstrated the presence of disturbances in skin microvascular reactivity in patients with T1DM, even in patients without any clinical signs of microangiopathy [148-154]. Santesson et al. conducted a longitudinal observational study of 17 patients with T1DM and found evidence of skin microvascular dysfunction evaluated with laser Doppler fluxmetry and post-occlusive reactive hyperemia (PORH) after 7-9 years of diabetes, which preceded the first identified case of retinopathy [155]. Thus, disturbances in skin microvascular reactivity seem to anticipate clinical microangiopathy in T1DM and may therefore be a useful tool for early detection of high-risk individuals.

Although type 1 diabetes is clearly associated with changes in skin microvasculature, the relationship between clinical microangiopathy and skin microvascular disturbances has not

been clearly established. It is important to note that the majority of studies have either not included any subjects with clinical microangiopathy [148, 152], not described the presence of clinical microangiopathy [149], or simply not correlated skin microvascular function to clinical microangiopathy [150, 151, 153]. Two studies to date, however, suggest that patients with clinical microangiopathy have more pronounced disturbances to skin microvascular reactivity than those without [154, 156], but none of them have compared skin microcirculation in patients with clinical microangiopathy of different severity.

1.6.2 Methods to study skin microcirculation

There are methods to study whole skin perfusion as well as individual capillary blood flow. Since skin perfusion is low at rest, vasodilatory stimuli are commonly used to assess the functionality and vasodilatory reserve of the skin microcirculation. Furthermore, whereas the skin blood flow at rest has high variability even during standardized conditions, increases in blood flow following provocation are typically more reproducible [91]. Common physiological stimuli include heat or PORH, where transient ischemia of the occluded tissue results in increased blood flow and shear stress, causing the endothelium to release NO, which in turn induces vasodilation [91]. An alternative approach is pharmacological provocation using iontophoresis to allow for non-invasive application of vasoactive substances into the skin [157, 158]. As shown in Figure 7 below, changes in skin microcirculation can be assessed either through a direct method, such as functional capillaroscopy which assesses blood cell velocity in a single capillary, or through indirect methods that estimate oxygenation, such as transcutaneous oxygen tension, or skin perfusion, such as laser Doppler fluxmetry.

1.6.2.1 Iontophoresis

Iontophoresis involves using a small electrical current to enhance the transdermal perfusion of vasoactive substances. Since vascular tone is dependent both on the production of vasoactive substances by the endothelial cells as well as the response of the smooth muscle cells, different pharmacological agents are used to stimulate different parts of the system [91]. Typically, the positively charged Acetylcholine (ACh) is used to assess endothelium-dependent vasodilation, which refers to endothelial cell modulation of smooth muscle response through pathways involving not only NO, but also prostaglandins, endothelial hyperpolarization and sensory nerves [157]. The negatively charged Sodium Nitroprusside (SNP) is used to study endothelium-independent vasodilation, as it acts directly on the smooth muscle cells to cause relaxation [157].

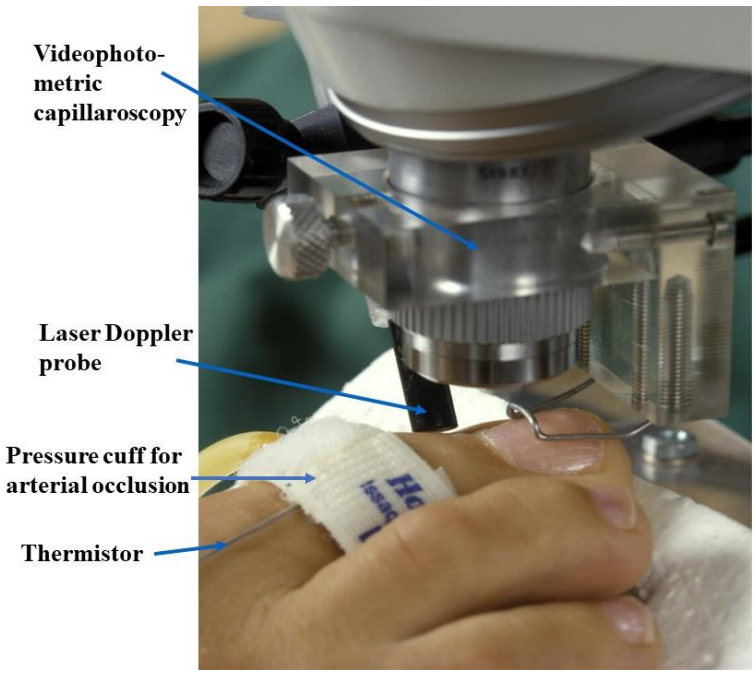


Figure 7: The setup for videophotometric capillaroscopy and laser Doppler fluxmetry of the skin microcirculation in the big toe.

Capillaroscopy is a direct method for detecting blood cell velocity in a single capillary, whereas laser Doppler fluxmetry is an indirect method for assessing whole skin perfusion. The thermistor registers skin temperature. Picture from Ph.D. thesis of Gun Jörneskog [159], reproduced with permission.

1.6.2.2 Laser Doppler fluxmetry and laser Doppler imaging

Laser Doppler fluxmetry involves a fiberoptic laser probe that generates a light with a specific wave-length, typically 780nm [157]. The light, when pointed at the skin, will be reflected back from moving blood cells in the skin microvasculature, causing a shift in frequency that depends on the number and velocity of the blood cells within the measured tissue volume [160], thus allowing for determination of whole skin perfusion in arbitrary units. Laser Doppler fluxmetry measures total skin microcirculation, i.e. both nutritional capillary blood flow and non-nutritional subpapillary blood flow. In most skin areas of the digits, more than 90% of the laser Doppler flux is generated in non-nutritional subpapillary vessels [161]. Laser Doppler imaging uses the same technique to assess skin perfusion in larger skin areas.

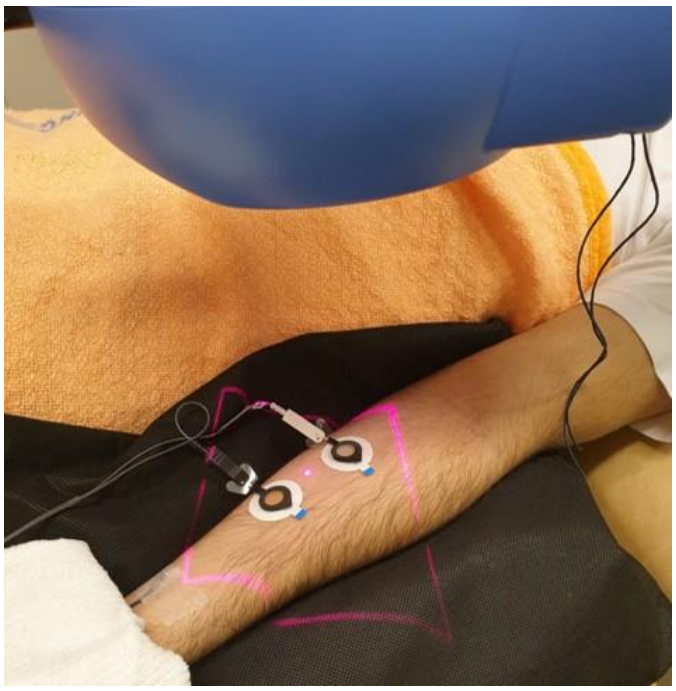


Figure 8: Laser Doppler fluxmetry measurements of the forearm following iontophoresis.

Iontophoresis is undertaken with acetylcholine (ACh) or sodium nitroprusside (SNP) to assess endothelium-dependent or endothelium-independent microvascular response, respectively. Picture taken by Sara Tehrani, reproduced with permission.

1.7 CLINICAL RATIONALE FOR THIS THESIS

Our current methods for detecting microvascular disease in type 1 diabetes remain insufficient, since we are often unable to identify clinical signs of microangiopathy until it is already well established. There is thus an urgent need to improve our understanding of the underlying pathogenic mechanisms as well as develop clinical biomarkers for early detection of microvascular damage to improve medical care for patients. MVs have emerged as key players in intercellular communication with potent proinflammatory and prothrombotic effects, but their exact role in microangiopathy in T1DM remains unclear. The skin microcirculation has also been shown to be a promising tool for studying microangiopathy, which may help us better understand the underlying disease mechanisms and aid in early detection of subclinical disease.

2 RESEARCH AIMS

2.1 AIMS

The overall aim of this thesis was to investigate how levels of proinflammatory and prothrombotic MVs as well as disturbances in skin microvascular function correlate with clinical microangiopathy in type 1 diabetes, in the hope of identifying potential biomarkers that can detect subclinical levels of microangiopathy.

Specific aims:

- To compare plasma levels of total MVs, platelet MVs and endothelial MVs, their expression of procoagulant PS and proinflammatory HMGB1, as well as MV-induced thrombin formation in-vitro, between patients with type 1 diabetes with and without clinical microangiopathy and matched healthy controls (**Papers I and II**).
- To study how different subpopulations of MVs differ between men and women with type 1 diabetes and healthy controls (**Paper I and II**).
- To evaluate the effect of different centrifugation protocols used in the preanalytical handling of MV samples on PMV and PS+ PMV levels measured by flow cytometry, as well as the influence on remaining cell fragments **(Paper III**).
- To elucidate how skin microvascular function relates to clinical microangiopathy in patients with type 1 diabetes (**Paper IV**).

3 MATERIALS AND METHODS

3.1 STUDY POPULATIONS AND OVERVIEW OF STUDY DESIGN

3.1.1 Papers I and II

Papers I and II are based on the same cross-sectional study in which 236 patients, 130 without any clinical microangiopathy (except for simplex or background retinopathy, an early and reversible stage of retinopathy) and 106 with microangiopathy (nephropathy, retinopathy, and neuropathy), were compared to 100 healthy controls matched for age, sex, and body mass index (BMI). Patients were recruited in 2009 from the diabetes outpatient clinic at the Department of Endocrinology and Diabetology, Danderyd Hospital, Stockholm, Sweden, and controls were recruited from the Stockholm population registry. To qualify for the study subjects had to be between 20-70 years of age. Exclusion criteria included (i) medical history of macrovascular disease, including heart disease, stroke and peripheral artery disease; (ii) present use of non-steroidal anti-inflammatory drugs or anticoagulants; and (iii) pregnancy. All controls were free from regular medications, with the exception of one individual who used a proton pump inhibitor daily. None of the female subjects used oral contraceptives.

For Paper I we analyzed total plasma MV levels and their expression of procoagulant PS. In a subgroup analysis we selected 25 patients with the most severe level of microangiopathy, 25 patients with no clinical signs of microangiopathy and 25 healthy controls and analyzed the functional ability of MVs isolated from the subjects to trigger thrombin generation in-vitro. For Paper II we looked at subtypes of MVs from different cellular origins, focusing on platelet and endothelial MVs, and studied their expression of procoagulant PS and proinflammatory HMGB1. We also looked at the correlation between MV levels and clinical patient characteristics as well as results from standard blood tests. Differences in MV levels between men and women with type 1 diabetes and healthy controls were compared for both papers.

3.1.2 Paper III

Our laboratory had been part of an on-going international collaboration to try to improve and standardize flow cytometry analysis between different laboratories worldwide. However, between the time that experiments for Papers I and II were carried out and the statistical analysis and interpretation of results were undertaken, further methodological developments had been made in the EV field. Consequently, in 2014, the International Society for Extracellular Vesicles released a set of guidelines entitled the Minimal Information for Study of Extracellular Vesicles (MISEV 2014) [162], which was again updated in 2018 (MISEV 2018) [103]. The updated MISEV 2018 guidelines endorsed the centrifugation protocol employed by the International Society on Thrombosis and Haemostasis, which attempted to completely remove platelets from plasma samples using two consecutive rounds of

centrifugation at 2500 g for 15 minutes prior to freezing [163, 164], in order to try to minimize platelet activation and ex-vivo release of EVs [165, 166]. For Paper III, our group therefore initiated a laboratory investigation in which we analyzed how different centrifugation protocols used to prepare plasma samples for flow cytometry analysis of MVs affect MV levels as well as the purity of samples. Eleven healthy volunteers (3 men, 8 women, ages 28-73) not currently using any medications were recruited for the study. Four different centrifugation protocols for fresh samples and three different centrifugation protocols for frozen/thawed samples were compared, as detailed below, including both the protocol employed by our laboratory and that recommended by MISEV [103]. We analyzed PMV levels, PS+ PMV levels, and expression of phalloidin, a cell fragment marker.

3.1.3 Paper IV

Paper IV was a cross-sectional study looking at skin microcirculation in patients with type 1 diabetes with a disease duration of at least 30 years. The study compared three different groups: (i) 30 patients with no clinical signs of microangiopathy except for background retinopathy (considered an early reversible stage); (ii) 31 patients with diagnosed clinical microangiopathy of different degrees; (iii) 31 healthy controls. All patients were recruited from the outpatient clinic at the Department of Endocrinology and Diabetology at Danderyd Hospital, Stockholm, Sweden. We conducted clinical investigations of skin microvascular function, as detailed below, and correlated findings with clinical microangiopathy including diabetic nephropathy, retinopathy and both peripheral sensory neuropathy and autonomic neuropathy. We also analyzed the association between the skin microcirculation and common clinical parameters as well as results from standard blood work.

3.2 LABORATORY INVESTIGATIONS

3.2.1 Blood- and urine sampling

3.2.1.1 Papers I and II

Subjects arrived at the laboratory in the morning, having fasted a minimum of 10 hours. To avoid hypoglycemia, patients with type 1 diabetes had been carefully instructed to take a reduced dose of insulin the evening before the study and were also asked to abstain from their usual morning insulin until after blood samples were drawn, at which point subjects were served a light standardized breakfast. Subjects rested for 20 minutes (min) before venous blood sampling was carried out using no or a minimal amount of stasis. Blood samples were drawn into citrated test tubes (0.109 M citrate concentration). Samples underwent centrifugation at 2000g at room temperature (RT) within 1 hour. Aliquots of 250 µL platelet-poor plasma (PPP) were frozen at -80˚C and thawed immediately prior to analysis. Urine samples were also obtained for control of albuminuria.

3.2.1.2 Paper III

Subjects arrived in the laboratory following an over-night fast. Venous blood samples were drawn from an antecubital vein using no stasis. Blood samples were collected into vacutainer tubes (Becton Dickinson) anticoagulated using trisodium citrate (0.129 mol/L, pH 7.4) in a 1:9 ratio of trisodium citrate: blood. The first 3 mL of blood were discarded. Collection tubes were gently inverted five times and then maintained upright until time of centrifugation. Freshly collected plasma samples were then handled using one of four different centrifugation protocols:

(a) 2500g for 15 min at RT, after which the supernatant was re-centrifuged at 2500g for 15 min (reference protocol recommended by MISEV 2018);

(b) 2000g for 20 min at RT (our laboratory protocol);

(c) 3200g for 20 min at RT;

(d) 3200g for 40 min at RT.

Following centrifugation, 500 µL aliquots of plasma were allotted into cryotubes. One aliquot from each of the centrifugation protocols detailed above was analyzed fresh within 1 hour for assessment of platelet levels prior to freezing, while the rest were frozen at -80˚C.

Frozen aliquots of plasma were thawed in a water bath at 37˚C for 5 min and handled according to one of three different pre-analytical protocols for frozen/thawed samples:

(i) Samples were analyzed directly after thawing (reference protocol recommended by MISEV 2018);

(ii) Centrifugation at 2000g for 20 min at RT. Supernatant re-centrifuged at 13 000g for 2 min prior to analysis;

(iii) Centrifugation at 2000g for 20 min at RT.

An overview of the different centrifugation protocols is detailed in Figure 9 below.

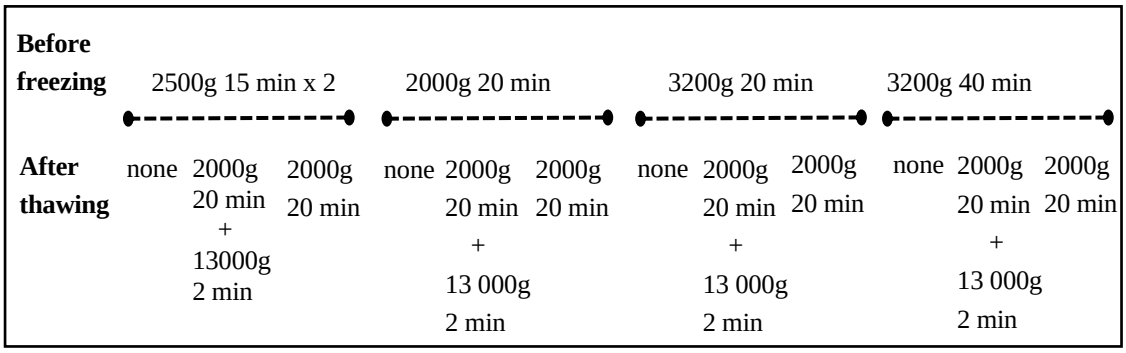


Figure 9: overview of the different centrifugation protocols of fresh and frozen/thawed samples compared in Paper III.

3.2.1.3 Paper IV

Subjects arrived at the laboratory in the morning, having fasted for at least 10 hours. They had been asked to abstain from smoking, snuff or working out in the morning prior to the investigations. To avoid hypoglycemia, patients with type 1 diabetes had been asked to reduce their insulin doses the evening before the study and refrain from insulin in the morning until after investigations had been completed. Brachial blood pressure was measured after 20 min of rest. Venous blood sampling was undertaken with no or minimal stasis. Urine samples were obtained for control of albuminuria. After blood/urine sampling as well as all investigations of skin microcirculation were complete, subjects were served a light standardized breakfast

3.2.2 Flow cytometry analysis of MVs (Papers I-III)

All flow cytometry analyses for this thesis were carried out using a Beckman coulter Gallios flow cytometer. Aliquots of PPP, previously frozen at -80˚C, were thawed in a water bath at 37˚C. Samples underwent centrifugation at RT, using a centrifugal force of 2000 g for 20 min. The supernatant underwent further re-centrifugation at 13 000g for 2 min. 20 µL of the supernatant was then incubated with fluorophore-coupled antibodies targeting the antigens of interest: 5 µL phalloidin-650 (Sigma-Aldrich St. Louise, MO, USA), 5 µL lactadherin-FITC (80 µM; Haemotologic Technologies, VT, USA), 5 µL CD42a-PE (Beckman Coulter, Brea, CA, USA), 5 µL CD61-PE (Abcam, Cambridge, UK), 5 µL CD144-APC (AH diagnostics, Stockholm, Sweden), 5 µL CD62E-APC (AH diagnostics, Stockholm, Sweden) and 5 µL CD14-APC (Beckman Coulter, Brea, CA, USA). These different markers are explained more in detail in Table 1 below.

MVs were identified by both their size (forward scatter) as well as complexity (side scatter) and defined as particles with a diameter < 0.9 µm. The setting of the MV-gate was decided on by a single skilled laboratory technician, who was blinded to the origin of the samples. Isotope controls and fluorescence compensation were carried out prior to analysis. The gate was

calibrated with the help of Megamix-plus FSC beads (BioCytex, Marseille, France) with diameters 0.1, 0.3, 0.5 and 0.9 µm (Figure 10).

Table 1. Proteins of interest studied using flow cytometry.

Protein / receptor	Significance
Phalloidin	Binds to exposed intracellular actin; cell fragment marker; samples with <10% considered sufficiently high quality [167]
Lactadherin	Binds negatively charged PS expressed on MVs, which helps propagate the coagulation cascade; marker of prothrombotic potential
CD42a, Glycoprotein IXa	Platelet MV marker
CD61, Integrin beta-three (also known as Glycoprotein IIIa)	Platelet MV marker
CD14, cluster of differentiation 14	Pattern recognition receptor expressed by monocytes as part of the innate immune system
CD144, VE-cadherin	Endothelial MV marker; located in tight junction between ECs
CD62E, E-selectin	Endothelial MV marker, released from activated ECs

CD, cluster of differentiation; PS, phosphatidylserine; MVs, microvesicles; ECs = endothelial cells.

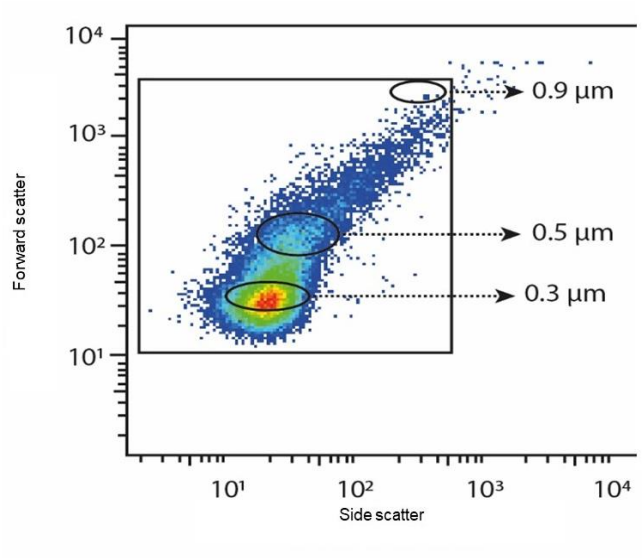


Figure 10. Microvesicle gating in flow cytometric analyses.

Representative dot-plot of microvesicle gating together with gates demonstrating beads with diameter 0.3, 0.5 and 0.9 µm.

A fluorescence minus one control was used for lactadherin. Results of the MV analyses are presented as total MV concentration (count per µL of original plasma sample). PS-expression was also assessed in terms of lactadherin mean fluorescence intensity (MFI). Both the intra-assay and inter-assay coefficients of variation (CV) for MV measurements at our lab were < 10%, calculated from 15 healthy volunteers on two consecutive days, with each lab result analyzed ten times in a row.

3.2.3 In-vitro thrombin generation induced by MVs (Paper I)

A subgroup analysis of the 25 patients with the most severe microvascular complications, 25 patients with no clinical microangiopathy, and 25 healthy controls was carried out to assess the ability of MVs to trigger thrombin generation in-vitro. The rationale was to isolate MVs from patients and controls and then add them to normal pooled plasma (NPP) to assess the pro-coagulant abilities of the MVs in and of themselves, using a standardized plasma environment.

First, pre-existing MVs were removed from NPP using high-speed centrifugation at 20 800g for 30 min at RT. MV-enriched pellets were then isolated from patient and control plasma samples. First, frozen 500 µL aliquots of PPP were thawed in a water bath at 37˚C. MVs were purified from the samples using high-speed centrifugation, where PPP was centrifuged at 2000 g for 20 min at RT, after which the supernatant (450 µL) was re-centrifuged at 20 800 g for 45 min at RT, in order to obtain a MV-enriched pellet. The supernatant (400 µL) was discarded and replaced by 400 µL of phosphate-buffered saline (PBS, pH 7.4), at which point the sample was re-centrifuged at 20 800g for an additional 45 min at RT. The resulting MV-enriched pellet was re-suspended in a buffer solution of 50 µL PBS.

The MV-enriched pellets isolated from patients and controls were added into three different samples: (i) NPP; (ii) NPP with MVs pre-incubated with lactadherin to block PS (16 µM, incubated at 20 min at RT); (iii) NPP containing corn trypsin inhibitor (CTI) to block the contact pathway (18.3 µg/mL).

Thrombin generation in vitro was measured using a modified version of the calibrated, automated thrombogram (CAT) assay, as previously detailed by Mobarrez et al. [168], without the addition of any other trigger of coagulation such as tissue factor or phospholipids. In brief, a fluorogenic substance that is selectively cleaved by thrombin was added to the samples, along with Fluca buffer solution. Fluorescence was measured at 30 second intervals for 60 min at 37˚C using a fluorometer. The variables obtained were as follows: (i) lag time, the time taken until the fluorescence signal deviates more than two standard deviations from the baseline; (ii) peak thrombin concentration; (iii) endogenous thrombin potential (ETP), the area under the concentration-time curve; (iv) time to peak, the time between the start of thrombin generation and the point at which the peak thrombin value is reached.

3.2.4 Biochemical analysis (Papers I, II and IV)

Standard blood tests including fasting plasma glucose (fP-glucose), creatinine, total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides and platelet-concentration were measured at the central hospital laboratory. The eGFR was calculated from creatinine using the revised Lund-Malmö equation, which has been validated for use in the Swedish population [169]. HbA_{1C} was measured using the Mono S method with high-performance liquid chromatography (Variant II; Bio-Rad Laboratories, Hercules, CA, USA), and values were converted to mmol/mol according to the International Federation of Clinical Chemistry standardization of HbA_{1C}. A particle-enhanced immunoturbidimetric method was used to analyze hsCRP, where a normal value is considered as $\leq$ 3mg/L (Beckman Inc., High Wycombe, UK). Endogenous plasma lactadherin levels (P-lactadherin) was measured using an enzyme-linked immunosorbent assay, ELISA, according to the specific instructions from the manufacturer (R&D systems, Minnesota, USA).

3.3 CLINICAL INVESTIGATIONS (PAPERS I, II AND IV)

Brachial blood pressure was taken as a mean of three readings from an oscillometric device (OMRON 705IT, OMRON Healthcare, Kyoto, Japan), measured in the supine position following 20 minutes of rest.

3.3.1 Microangiopathy scores

3.3.1.1 Papers I and II

The prevalence of clinically diagnosed microangiopathy (retinopathy, nephropathy and/or neuropathy) was assessed by a single skilled individual using information available in the electronic medical records of all subjects. Diabetic retinopathy of patients was assessed by funduscopic findings by ophthalmologists and categorized into three different categories: (i) no retinopathy except for simplex retinopathy, an early stage of retinopathy considered reversible; (ii) mild-moderate retinopathy; (iii) severe retinopathy, defined as either laser-treated non-proliferative diabetic retinopathy (NPDR) or proliferative diabetic retinopathy (PDR). Nephropathy was defined as either the prevalence of albuminuria on urinary dipstick tests (Clinitek®, Bayer Healthcare LLC, USA) on at least two occasions, or an eGFR below 60 mL/min/1.73 m^2. Microalbuminuria (in updated nomenclature referred to as moderately increased levels of albuminuria [60]) was defined as urinary ACR of 3.4-33.9 mg/mmol and macroalbuminuria (severely increased levels of albuminuria [60]) was defined as urinary ACR $\geq$ 34.0 mg/mmol. Neuropathy status was assessed bedside by physicians as part of the patients´ routine clinical care and defined as either a decreased light-touch perception upon testing with monofilament (Semmes-Weinstein 5.07), a diminished vibratory perception tested using a 128 Hz tuning fork, and/or decreased patellar or achilles reflexes. Autonomic neuropathy was not assessed for the purpose of these two papers.

3.3.1.2 Paper IV

Information about diabetic retinopathy was obtained from the medical records based on the fundoscopic reports from ophthalmologists at regular intervals as part of patients´ routine medical care. Retinopathy was categorized into four different stages according to the International Clinical Disease Severity Scale for Diabetes Retinopathy [170]: (i) no retinopathy; (ii) mild NPDR (background retinopathy), defined by presence of dot bleedings or a few microaneurysms; (iii) moderate to severe NPDR, identified by soft and hard exudates, venous beadings and/or intraretinal microvascular abnormalities; (iv) PDR, defined by neovascularization, hemorrhages and/or retinal detachment or previous laser-treatment.

Diabetic nephropathy was categorized into four stages based on the medical records: (i) no albuminuria; (ii) microalbuminuria (moderately increased albuminuria), defined as urinary ACR 3.4-33.9 mg/mmol in at least two morning samples or albuminuria of 30-300 mg in a 24-hour urine collection; (iii) macroalbuminuria (severely increased albuminuria), defined as urinary ACR $\geq$ 34 mg/mmol in at least two morning samples or albuminuria >300 mg in a 24-hour urine collection; (iv) chronic kidney disease, defined as at least moderate chronic kidney disease stage 3B with eGFR < 45 mL/min/1.73m^2.

Diabetic neuropathy in this study included both distal sensory neuropathy in the lower extremities, assessed according to the American Diabetes Association guidelines 2005 [171], as well as autonomic neuropathy. Vibration perception was assessed using a 128 Hz tuning fork at the dorsum of the interphalangeal joint of the big toe, comparing the patients´ sensation with that of an examination with the tuning fork instead placed at the dorsal wrist. Superficial perception was examined using a 5.07 Semmes-Weinstein monofilament placed both at the plantar surface of the big toe and at the center of the heel, with the test being repeated three times. Autonomic neuropathy for the purpose of the study included mentions of gastroparesis and/or erectile dysfunction in the medical records. Other potential manifestations of autonomic neuropathy, such as postural hypotension, were not included. Microangiopathy across the different vascular beds was added as described in Table 2 below, such that all patients were given a score from 0 to 9 points.

3.3.2 Investigations of skin microvascular function (Paper IV)

3.3.2.1 Iontophoresis and Laser Doppler imaging

Skin microcirculation was assessed using Laser Doppler perfusion imaging (PeriScan PIM II; Perimed, Järfälla, Sweden) following iontophoresis, a non-invasive method of applying drugs across the skin with the aid of a small electric current.

Table 2. Clinical microangiopathy score used in Paper IV.

	Degree of clinical microangiopathy	Points
Retinopathy	No signs of retinopathy	0
	Background retinopathy	1
	Non-proliferative retinopathy	2
	Proliferative retinopathy	3
Nephropathy	No proteinuria	0
	Microalbuminuria	1
	Macroalbuminuria	2
	Chronic kidney disease *	3
Neuropathy	No signs of neuropathy	0
	Reduced sense of vibration	1
	Reduced superficial sensation	1
	Autonomic dysfunction	1

* Chronic kidney disease stage 3B or higher, defined as estimated glomerular filtration rate (eGFR) < 45 mL/min/1.73m^2

Acetylcholine (ACh, diluted in deionized water at 2%; Sigma-Aldrich AB, Stockholm, Sweden) was used to trigger endothelium-dependent microvascular reactivity and sodium nitroprusside (SNP, diluted in deionized water at 2%; Hospira, Inc., Lake Forest, IL, USA) was used to assess endothelium-independent microvascular reactivity. Electrode chambers (LI611 Drug Delivery Electrode Imaging; Perimed, Järfälla, Sweden) filled with a small amount of either ACh or SNP were positioned at the volar side of the left forearm, taking care to avoid any injured skin, hair, or perceivable veins. An iontophoresis controller (Perilont 382b; Perimed, Järfälla Sweden) then supplied a direct current of 0.1 mA for 60 seconds. An anodal charge was used to deliver the ACh and a cathodal charge delivered the SNP. Skin microvascular flux, expressed in arbitrary units (AU), was assessed using laser Doppler imaging, with each image consisting of data gathered from approximately 150 measuring points for 36 seconds. Data was recorded for 10 minutes following iontophoresis with ACh and 14 minutes for iontophoresis with SNP. The mean CV for peak microvascular flux in our laboratory was 11% for iontophoresis of ACh and 20% for iontophoresis of SNP, calculated from seven healthy individuals assessed on three separate days.

3.3.2.2 Nailfold capillaroscopy

Capillary blood flow at rest and following arterial occlusion was assessed by analyzing nailfold capillaries of the big toe using a computerized videophotometric cross-correlation technique (CapiFlow AB®, Stockholm, Sweden). Capillary blood cell velocity (CBV) was measured at rest, during and following a 1-minute arterial occlusion, brought on by inflating a miniature cuff at the base of the proximal phalanx of the digit to a cuff pressure of 200 mm Hg. The

following parameters were analyzed: CBV rest value (mm/s); CBV peak (mm/s) following 1-minute arterial occlusion; CBV ttp, time to peak (s); CBV porh, post-occlusive reactive hyperemia, calculated as the % increase of CBV from rest to peak flow. The temperature of the skin of the nailfold under investigation was continuously measured using an electronic thermistor (Exacon, Copenhagen, Denmark).

To get reliable interpretation of data, only subjects whose capillaries generated good optical signals, defined as clearly visible blood cell movements and plasma gaps, were included in the analysis. Subjects whose capillaries were not clearly visualized due to thick or cracked skin or deformities of the toe were excluded. The CV for repeated measurements of CBV peak and CBV ttp were 13% and 11% at our laboratory [172].

3.3.2.3 Laser Doppler Fluxmetry

The total skin microcirculation was measured in the big toe using Laser Doppler fluxmetry (LDF) (Periflux, Perimed, Järfälla, Sweden) simultaneously as the measurements of capillary blood flow with capillaroscopy. The laser probe was positioned on the skin nailfold of the big toe, as close as possible to the microscopic field of view. Values obtained were LDF rest value (arbitrary units, AU); LDF peak flow (AU) after a 1-minute arterial occlusion with a miniature pressure cuff placed at the proximal phalange of the toe and inflated to 200 mm Hg; LDF ttp, time to peak (s); LDF porh, post-occlusive reactive hyperemia, the percentage increase between LDF rest value and LDF peak flow (%). AU obtained by LDF are proportional to the average velocity of blood cells in relation to their concentration within the volume of blood measured [173]. Subjects with skin that was too thick or damaged to achieve a proper LDF signal were excluded.

3.4 STATISTICAL ANALYSIS

3.4.1 Sample size calculations

The size of the study population used in Papers I and II was originally determined based on power calculations for a study to detect differences in fibrin clot characteristics between women and men with type 1 diabetes [47]. However, in planning this thesis, post hoc power analysis using 2-sided t-tests showed that the size of the study population was sufficiently large to be able to detect a 20% difference in the level of total plasma MVs as well as PS+ MVs between patients with type 1 diabetes and controls with a power of 80% and alfa level of 0.05. The subgroup analysis in Paper I was powered to detect a 20% difference in ETP between patients with and without microangiopathy (power 80%, alfa level 0.05).

For Paper IV, power size calculation using a 2-sided t-test showed that 27 patients in each group was necessary in order to detect a 25% difference in ACh-mediated peak microvascular

flux between patients with and without clinical microangiopathy (power 80%, alfa level 0.05), and we therefore chose to include at least 30 subjects per group.

3.4.2 Statistical analysis of data

Raw data are presented as either numbers or percentages. Calculated results are presented as means ± standard deviations (SD) or 95% confidence intervals (C.I.) for normally distributed data, and medians with interquartile ranges (IQR) (i.e. 25^{th} to 75^{th} percentiles) for skewed data. Raw data was checked for normality using Shapiro-Wilks tests. Skewed data was log-transformed and again checked for normality before choosing the appropriate statistical method for analysis. For normally distributed data, differences between groups were calculated using either independent t-tests or one-way analysis of variances (ANOVA) with contrast analysis. Analysis of covariance (ANCOVA) was used to compare groups while controlling for potential confounding factors (covariates). In the case of Paper IV, repeated measures ANOVA was used to analyze the results of the skin microcirculation data that had been collected at several time-points. For data that remained skewed after log transformation, Mann-Whitney U or Kruskal-Wallis ANOVA with contrasts were used. Correlations between variables were analyzed using simple regression. Results were considered statistically significant if $p < 0.05$. All statistical analysis was carried out using Statistica version 13 (TIBCO Software Inc.).

3.5 ETHICAL CONSIDERATIONS

The studies in this thesis were all conducted in accordance with the Declaration of Helsinki and ethical permits were obtained by the local ethics board. Written informed consent was obtained by all subjects.

Several important ethical principles are affected by these studies. First, the principle of informed consent. All subjects in our studies were given both oral and written information before being asked whether they would want to participate. We felt that by giving the information in two different ways, the ability of the participants to make a fully informed choice was increased. Subjects were also informed of their right to withdraw at any time without the need to give an explanation.

Another important ethical principle is that of personal integrity. We were expressively given permission from patients to access information in their electronic medical records, for the purpose of relating MV levels and skin microcirculation to microvascular complications. However, after collection of data in the medical records the data was then anonymized, and national identity numbers and names were replaced by study ID numbers for the purpose of all future analysis. Also, all data was analyzed only at the aggregate level by group, which served to further improve the anonymity of patients.

Potential discomfort caused by venipuncture in our studies is temporary and likely to at most cause some bruising that should only last a few days. The clinical investigations including control of blood pressure and measurements of skin microvasculature are all non-invasive and not painful.

Ethically speaking it is of utmost importance that we strive to use the results of our research to do good and to not cause any harm to our patients. Patients with type 1 diabetes are disproportionately affected by microvascular complications, and there is an imperative need to improve the detection of microangiopathy as well as risk-stratification of individual patients to be able to optimize medical treatment. We are therefore of the opinion that the potential benefit to patients included in our studies far outweigh any potential risks.

The ethics permits for the different studies are listed below:

- Papers I and II: Dnr 2008/61-31/4 (healthy controls) and Dnr 2009/281-31/4 (patients); additional amendments 2016/2428-32; 2018/81-32.
- Paper III: Dnr 2017/2190-31.
- Paper IV: Dnr 03-499; additional amendments 2009/1512-32; 2012/1547-32; 2017/568-32.
- Unpublished data – same ethics permits as for Papers I, II and IV.

4 RESULTS –

4.1 BASELINE CHARACTERISTICS OF THE STUDY POPULATIONS

4.1.1 Papers I and II

The baseline characteristics of the subjects included in the first two papers are detailed in Table 3 below. Patients and healthy controls did not differ with regard to age, proportion of men versus women, BMI, tobacco use, or eGFR. Patients had significantly higher plasma glucose levels, systolic blood pressure, and platelet levels than controls but lower diastolic blood pressure ($p < 0.001$ for all). For blood lipids, patients had lower total cholesterol and LDL but higher HDL ($p < 0.001$ for all). Patients tended to have higher hsCRP, but this did not quite reach statistical significance ($p = 0.051$). Comparing the two patient groups, patients with microangiopathy were older, had a longer diabetes duration, higher systolic blood pressure, higher triglyceride levels, lower eGFR, and worse glycemic control. Patients with microangiopathy had greater use of angiotensin converting enzyme inhibitors (ACE-i), angiotensin II receptor blockers (ARBs), statins, and insulin pumps than patients without microangiopathy.

4.1.2 Paper IV

Subject characteristics of patients and controls are detailed in Table 4 below. Patients with T1DM did not differ in age, BMI, eGFR, or percentage of women compared to healthy controls. There was a tendency towards higher tobacco use among patients, but this did not reach statistical significance ($p = 0.08$). Patients had higher systolic blood pressure, lower total cholesterol, LDL and triglycerides but higher HDL compared to controls ($p < 0.05$ for all). There was no difference in fP-glucose, diabetes duration or tobacco use between the two patient groups. Patients with microangiopathy had higher BMI, higher systolic blood pressure, lower eGFR, lower HDL and higher triglyceride levels compared with patients without microangiopathy.

4.2 PATIENTS WITH TYPE 1 DIABETES HAVE SIGNIFICANTLY ELEVATED MV LEVELS OF ALL SUBCLASSES (PAPERS I AND II)

The most abundant MVs in plasma amongst all subjects were of platelet origin, as demonstrated using both CD42a and CD61 as markers (median 31% (25-41) and 26% (21-33) respectively). MVs derived from monocytes (CD14+) comprised 8% (5-15%), while endothelial cell markers CD62E (E-selectin) and CD144 (VE-cadherin) were found on 5% (4-11%) and 2% (0.4-3%, respectively. Differences between the patients and controls were most pronounced for endothelial and monocyte MVs, as shown in Table 5. below.

Table 3. Characteristics of study population in Papers I and II.

	Patients without MA (n = 130)	**Patients with MA (n = 106)**	**Healthy controls (n = 100)**	**P-value patients versus controls**	**P-value between patient groups**
Age (years)	42 ± 13	48 ± 13	45 ± 10	0.86	**< 0.001**
Women (n, %)	62 (48)	45 (43)	46 (46)	0.91	0.42
BMI (kg/m^2)	24.6 ± 3.5	25.5 ± 4.0	25.0 ± 3.1	0.93	0.06
Tobacco user[a] (n, %)	36 (28)	30 (29)	20 (22)	0.26	0.84
SBP (mmHg)	124 ± 14	133 ± 19	121 ± 11	**< 0.001**	**< 0.001**
DBP (mmHg)	72 ± 8	74 ± 9	78 ± 8	**< 0.001**	0.16
Diabetes duration (years)	18 ± 13	28 ± 13	n/a	n/a	**< 0.001**
HbA_{1C} (mmol/mol)	58 (51 - 68)	64 (55 - 72)	not measured	n/a	**0.003**
fP-glucose (mmol/L)	9.7 ± 4.3	10.9 ± 4.7	5.1 ± 0.5	**< 0.001**	**0.04**
Lipids (mmol/L)					
- Cholesterol	4.4 (4.0 - 4.9)	4.5 (4.0 - 5.1)	5.1 (4.6 - 5.6)	**< 0.001**	0.15
- LDL	2.5 (2.1 - 2.9)	2.6 (2.1 - 3.0)	3.3 (2.8 - 3.8)	**< 0.001**	0.42
- HDL	1.6 (1.2 - 1.8)	1.4 (1.2 - 1.8)	1.3 (1.0 - 1.6)	**< 0.001**	0.22
- Triglycerides	0.6 (0.5 - 0.8)	0.7 (0.6 - 1.0)	0.7 (0.5 - 1.2)	0.11	**0.001**
eGFR ($mL/min/1.73m^2$)	87 ± 12	83 ± 14	87 ± 10.1	0.29	**0.003**
Platelet count ($x10^9/L$)	223 (190 - 248)	221 (199 - 261)	206 (182 - 239)	**0.001**	0.10
hsCRP (mg/L)	0.9 (0.5 - 1.9)	1.0 (0.4 - 2.5)	0.7 (0.4 - 1.6)	0.05	0.39
Microangiopathy (n,%)					
- Retinopathy	not present	84 (79)	n/a	n/a	n/a
- Nephropathy		29 (27)			
- Neuropathy		43 (41)			
Medications (n, %)					
- ACE-i/ARBs	21 (16)	46 (43)			**< 0.001**
- Betablockers	3 (2)	12 (11)	none	n/a	-
- Calcium antagonists	3 (2)	9 (9)			-
- Statins	34 (26)	51 (48)			**< 0.001**

Data presented as mean ± standard deviation (SD), median with interquartile range (IQR), numbers (n) or percentages (%). P-values are based on one-way ANOVA or Kruskal-Wallis ANOVA with contrast analysis. Statistically significant differences ($p < 0.05$) are highlighted in bold. [a] Current smoker or snuff user. Abbreviations used: MA, microangiopathy; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA_{1C}, glycated hemoglobin; fP, fasting plasma; LDL, low-density lipoprotein; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate; hsCRP, highly sensitive C-reactive protein; ACE-i, angiotensin converting enzyme inhibitors; ARBs, angiotensin II receptor blockers; n/a, not applicable.

Total circulating MVs were significantly higher in patients with type 1 diabetes compared to controls ($p < 0.001$, Figure 11), which remained after controlling for differences in blood pressure between the groups (data not shown). While PS+ MV levels as well as PS-expression on MVs measured using lactadherin MFI was higher in patients than in controls ($p < 0.001$ for both, Figures 11 and 18), the largest contribution to the increased MV levels in patients came from PS-negative MVs (Figure 11). Out of the total MV population, 31% (25-40) were PS-positive amongst patients, compared to 44% (43-47) among healthy controls ($p < 0.001$).

Table 4. Characteristics of study population in Paper IV.

	Patients without MA n = 30	Patients with MA n = 31	Healthy controls n = 31	P-value patients versus controls	P-value between patient groups
Age (years)	54 ± 10	56 ± 14	56 ± 13	0.72	0.55
Women (n, %)	18 (60)	16 (52)	16 (52)	0.83	0.61
BMI (kg/m^2)	23.4 ± 4.2	25.9 ± 3.8	24.6 ± 3.0	0.95	**0.01**
Tobacco user[a] (n, %)	4 (13)	9 (29)	2 (6)	0.08	0.21
SBP (mmHg)	129 ± 13	144 ± 22	125 ± 16	**0.004**	**0.001**
DBP (mmHg)	71 ± 8	73 ± 12	73 ± 9	0.53	0.41
Diabetes duration (years)	41 ± 11	40 ± 13	-	-	0.57
HbA_{1C} (mmol/mol)	55 ± 8	66 ± 13	-	-	< **0.001**
fP-glucose (mmol/L)	10.3 (8.5 – 12.9)	9.9 (6.9 – 13.6)	5.4 (5.0 – 5.8)	< **0.001**	0.74
Lipids (mmol/L)					
- Cholesterol	4.5 (4.1 – 5.1)	4.4 (3.9 – 5.0)	5.0 (4.6 – 5.5)	< **0.001**	0.33
- LDL	2.5 (2.0 – 2.6)	2.4 (1.9 – 2.9)	3.4 (2.9 – 3.9)	< **0.001**	0.98
- HDL	1.7 (1.4 – 1.9)	1.4 (1.2 – 1.7)	1.3 (1.1 – 1.6)	**0.004**	**0.02**
- Triglycerides	0.5 (0.4 – 0.8)	0.9 (0.6 – 1.2)	0.9 (0.6 – 1.1)	**0.04**	< **0.001**
eGFR ($mL/min/1.73\ m^2$)	78 ± 8	65 ± 20	73 ± 13	0.92	**0.001**
Medications (n,%)					
- ACE-i or ARBs	8 (27)	21 (68)	-	-	**0.006**
- Betablockers	1 (3)	8 (26)	-	-	0.13
- Calcium antagonists	1 (3)	11 (35)	-	-	**0.03**
- Statins	8 (27)	17 (55)	-	-	0.06

Data presented as mean ± standard deviation (SD), median with interquartile range (IQR), numbers (n) or percentages (%). P-values are based on contrasts between patients and controls or patient groups in one-way ANOVA. [a] Current smoker or snuff user. Abbreviations used: MA, microangiopathy; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA_{1C}, glycated hemoglobin; fP, fasting plasma; LDL, low-density lipoprotein; HDL, high-density lipoprotein; eGFR, estimated glomerular filtration rate; ACE-i, angiotensin converting enzyme inhibitors; ARBs, angiotensin II receptor blockers.

Table 5. The percentage of total MVs that expressed different cell-specific markers.

	Patients (% of total MVs)	Controls (% of total MVs)
CD42a+ (glycoprotein IX, platelet marker)	28 (23 - 38)	39 (32 - 45)
CD61+ (integrin beta 3/glycoprotein IIIa, platelet marker)	24 (19 - 33)	29 (25 - 33)
CD62E+ (E-selectin, endothelial marker)	8 (4 - 14)	0.2 (0.1 - 0.4)
CD144+ (VE-cadherin, endothelial marker)	2 (1 - 4)	0.2 (0.1 - 0.4)
CD14+ (monocyte marker)	13 (7 - 17)	5 (4 - 6)

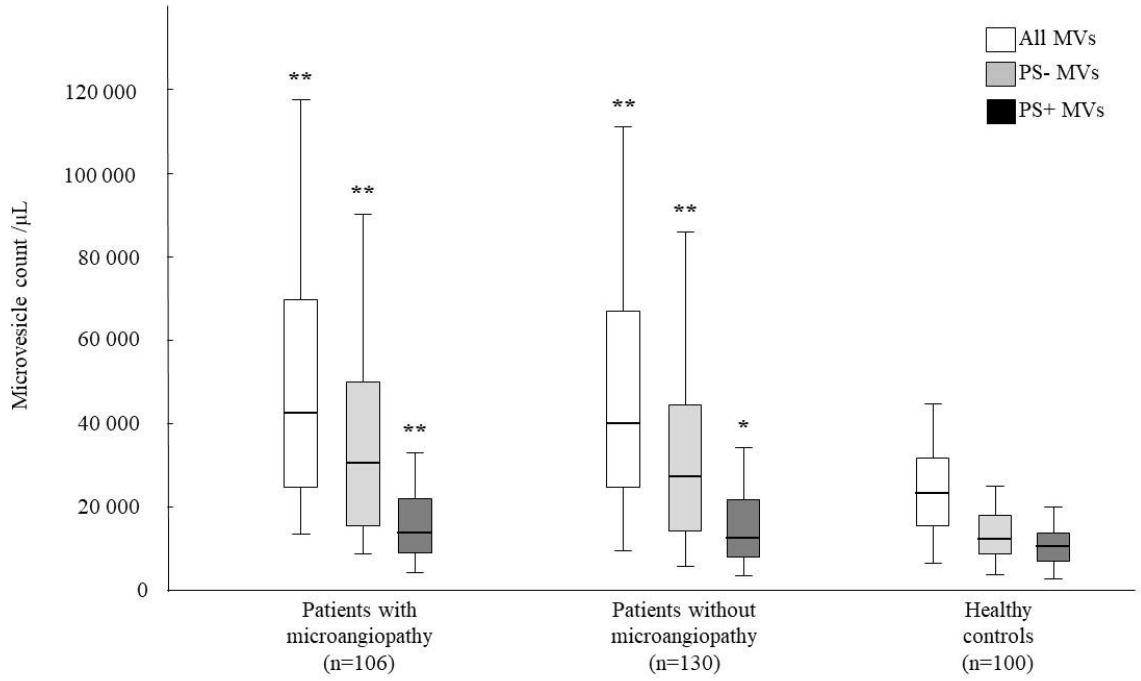


Figure 11. Total plasma microvesicles in patients with type 1 diabetes and controls. Total plasma levels of microvesicles (MVs) and those expressing phosphatidylserine (PS) as well as those not expressing PS were all significantly higher in patients with type 1 diabetes than in controls. No significant differences in MV subpopulations were found between patients with and without microangiopathy. Data is presented as median, interquartile range (boxes) and 95% confidence interval (whiskers). *p = 0.003 and **p < 0.001 compared to healthy controls (one-way ANOVA with contrast analysis of logged data).

Looking at MVs of different cellular origin, patients with type 1 diabetes had significantly higher levels of EMVs of all subclasses compared to healthy controls, as shown in Figures 12 and 13. Similarly, patients had elevated levels of PMVs subpopulations compared to controls, as shown in Figures 14 and 15. An ANCOVA model was also employed to compare MV subpopulations between patients and controls while correcting for tobacco use as well as significant differences in subject baseline characteristics including fP-glucose, systolic- and diastolic blood pressure, total cholesterol, LDL, HDL, plasma platelet concentration, and the use of medications including ACE-i, ARBs, statins, and insulin treatment modality. All significant differences remained (data not shown).

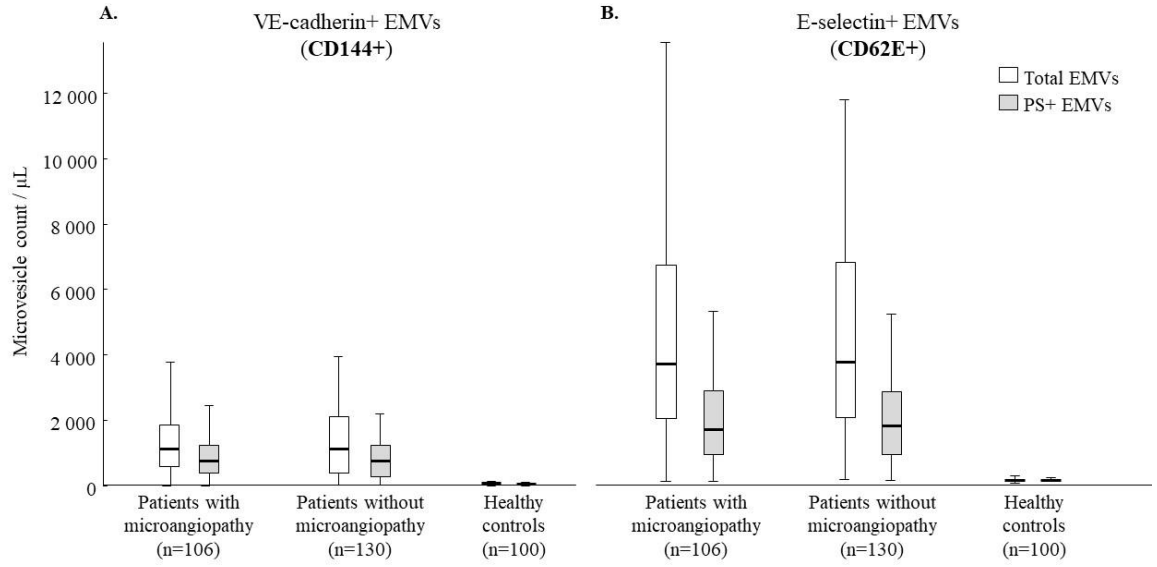


Figure 12. Endothelial microvesicles in patients with type 1 diabetes and controls. Patients with type 1 diabetes had significantly higher plasma levels of total endothelial microvesicles (EMVs) and EMVs expressing phosphatidylserine (PS) compared to healthy controls ($p < 0.001$ for all, ANOVA with contrast analysis), whereas patients with and without microangiopathy did not differ significantly in EMV levels. Data is presented as median, interquartile range (boxes) and non-outlier range (whiskers).

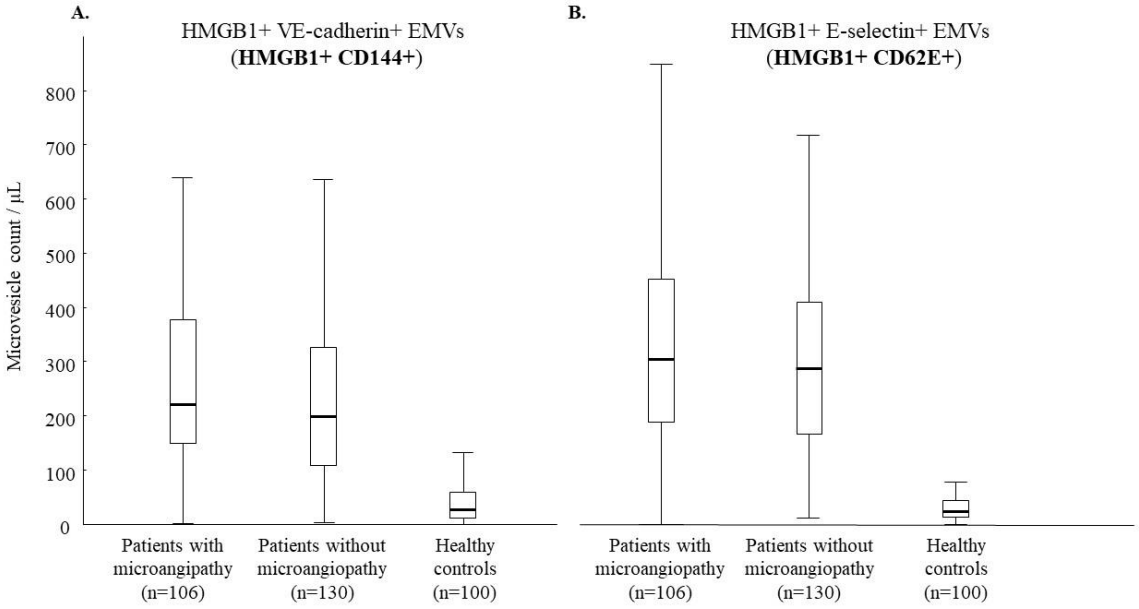


Figure 13. HMGB1+ endothelial microvesicles in patients with type 1 diabetes and controls.
Patients with type 1 diabetes had significantly higher plasma HMGB1+ EMV levels compared to healthy controls ($p < 0.001$ for all), whereas patients with and without microangiopathy did not differ. Figure shows median, interquartile range (boxes) and non-outlier range (whiskers). HMGB1, high-mobility group box-1 protein.

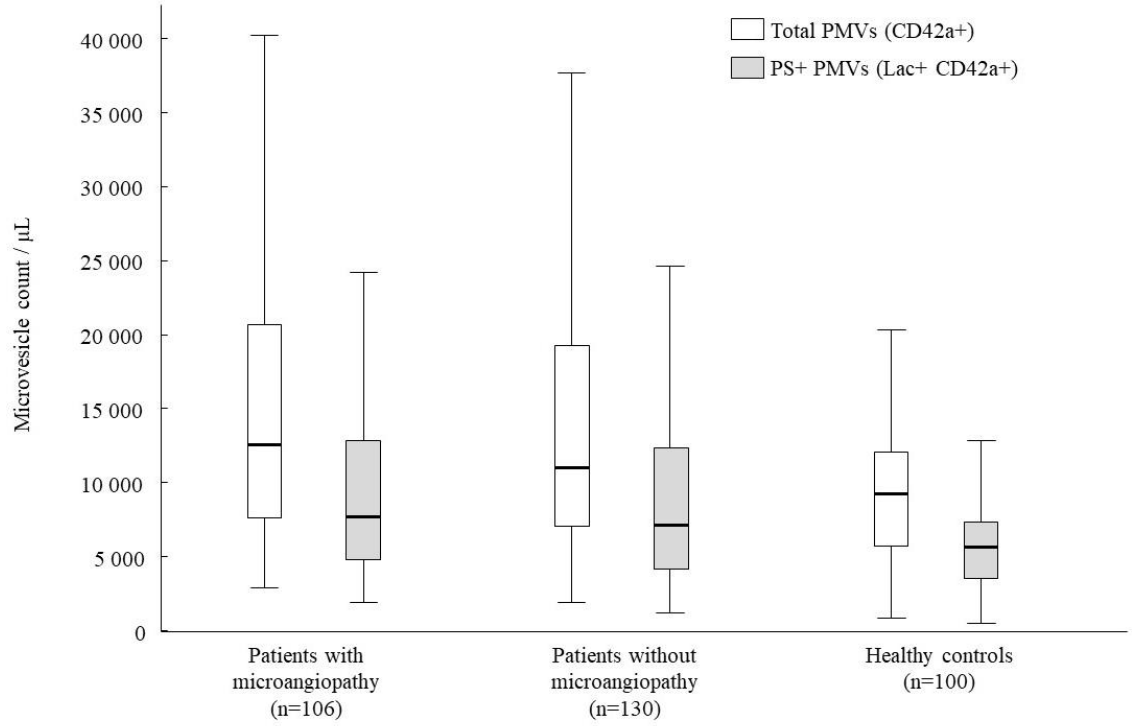


Figure 14. Platelet microvesicles in patients with type 1 diabetes and controls.
Patients with type 1 diabetes had higher plasma levels of platelet microvesicles (PMVs) and PMVs expressing phosphatidylserine (PS) compared to healthy controls ($p < 0.001$ for all, ANOVA with contrast analysis), whereas patients with and without microangiopathy did not differ significantly. Figure shows median, interquartile range (boxes) and non-outlier range (whiskers). CD42a, glycoprotein IX; Lac, lactadherin.

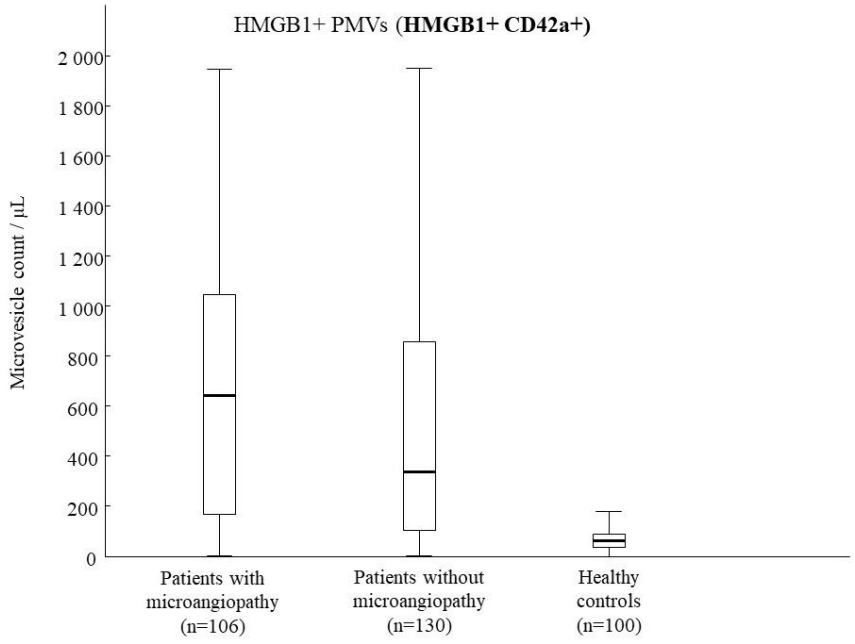


Figure 15. HMGB1+ platelet microvesicles in patients with type 1 diabetes and controls.
Patients had higher plasma HMGB1+ platelet microvesicles (PMVs) compared to healthy controls ($p < 0.001$, ANOVA with contrast analysis). Patients with microangiopathy were initially found to have higher levels of HMGB1+ PMVs than patients without microangiopathy ($p = 0.04$), but this difference did not remain in ANCOVA analysis controlling for differences in baseline characteristics between patient groups. Figure shows median, interquartile range and non-outlier range. HMGB1, high mobility group box-1 protein. CD42a, glycoprotein IX.

4.3 PLASMA MICROVESICLES IN TYPE 1 DIABETES DO NOT CORRELATE WITH CLINICAL MICROANGIOPATHY (PAPERS I AND II)

Patients with microvascular disease were initially shown to have significantly higher levels of HMGB1+ CD42+ PMVs compared to patients without microvascular complications ($p = 0.04$, ANOVA with contrast analysis). After controlling for relevant background characteristics that differed significantly between patients with and without complications, however, this difference was no longer statistically significant ($p = 0.42$, ANCOVA analysis controlling for age, systolic blood pressure, diabetes duration, eGFR, fP-glcuose, HbA_{1C}, ACE-i, ARBs, statins, insulin treatment modality). For all of the other MV subpopulations measured, there were no significant differences between patients with and without clinical microangiopathy in either ANOVA analysis or ANCOVA analysis correcting for the covariates mentioned above (data not shown). MV subpopulations were also analyzed in relation to the severity of microangiopathy, with no significant findings (data not shown). A smaller subgroup analysis was also carried out to compare MV subpopulations in the 25 patients with the most pronounced microangiopathy compared to 25 patients without microangiopathy, but again no statistically significant differences were found (data not shown).

4.4 WEAK CORRELATIONS BETWEEN MV SUBPOPULATIONS AND PATIENT CHARACTERISTICS (PAPERS I AND II)

Total MV levels had a weak positive correlation to systolic blood pressure ($r = 0.27$, $p < 0.001$). No significant correlations were found between total MVs, total PS+ MVs or total PS- MVs and other clinical parameters of interest, including fP-glucose, HbA_{1C}, diabetes duration, BMI or lipid levels. Simple regression analysis was also carried out to study the correlation between these different clinical parameters and EMV/PMV subpopulations. HMGB1+ E-selectin+ EMVs had a positive correlation with LDL-levels among patients ($r = 0.32$, $p < 0.001$). There was also several other very weak associations between MV subpopulations and different clinical characteristics, but without any consistent pattern amongst patients and controls, and all had a r-value below 0.3 (data not shown).

4.5 MICROVESICLES FROM PATIENTS INDUCE FASTER THROMBIN GENERATION, BUT NO DIFFERENCE IN TOTAL AMOUNT (PAPER I)

In-vitro thrombin generation induced by MVs isolated from patients with type 1 diabetes and healthy controls was measured using CAT, as shown in Figure 16 below. Patients had shorter lag time (17 ± 4 min vs. 20 ± 4 min) and time to peak (26 ± 4 min vs. 29 ± 4 min) compared to healthy controls ($p = 0.01$ and $p = 0.006$ respectively, ANOVA with contrast analysis). Peak thrombin levels and ETP did not differ between patients and controls ($p = 0.08$ and $p = 0.31$). No differences between patients with and without microangiopathy or between men and women with or without diabetes were detectable for any of the thrombin generation parameters (data not shown).

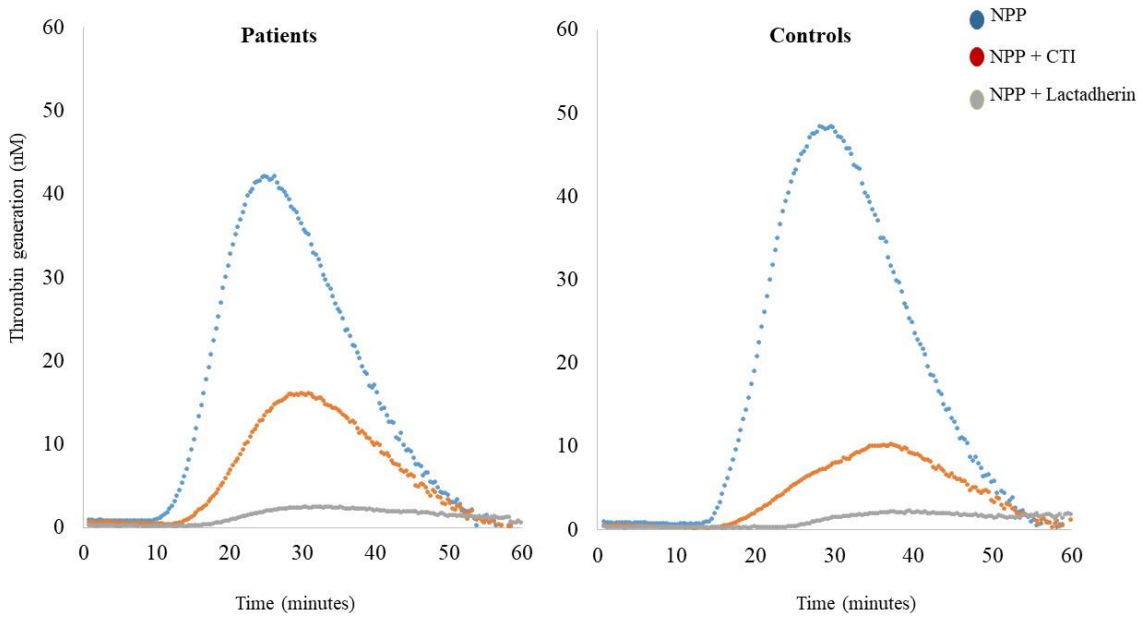


Figure 16. Thrombin generation induced by microvesicles from patients with type 1 diabetes and controls.

Microvesicles (MVs) isolated from patients with type 1 diabetes and healthy controls were isolated and added to normal-pooled plasma (NPP, blue dots), NPP + corn trypsin inhibitor (CTI, red dots) and NPP + lactadherin (gray dots). MVs from patients resulted in faster thrombin generation ($p < 0.05$), but the total amount of thrombin (ETP) did not differ. In samples where lactadherin was added to block PS, there was a significant decrease in all thrombin generation parameters and almost no thrombin was generated. Blocking the contact pathway through addition of CTI to NPP also resulted in a reduction of thrombin generation, but the effect was not as pronounced as for lactadherin.

4.6 P-LACTADHERIN LEVELS CORRELATE WITH CLINICAL MICRO-ANGIOPATHY (PAPER I)

Patients with type 1 diabetes had elevated P-lactadherin levels compared to healthy controls, with significantly higher P-lactadherin levels amongst patients with microangiopathy compared to those without, as shown in Figure 17. There was no correlation between proportion/numbers of PS+ MVs and P-lactadherin. In our study, P-lactadherin had a weak positive correlation to fP-glucose ($r = 0.16$, $p = 0.01$), HbA_{1C} ($r = 0.15$, $p = 0.02$), LDL ($r = 0.20$, $p = 0.003$) and total cholesterol ($r = 0.34$, $p < 0.001$), but no significant associations to BMI, age, diabetes duration, blood pressure, hsCRP or eGFR.

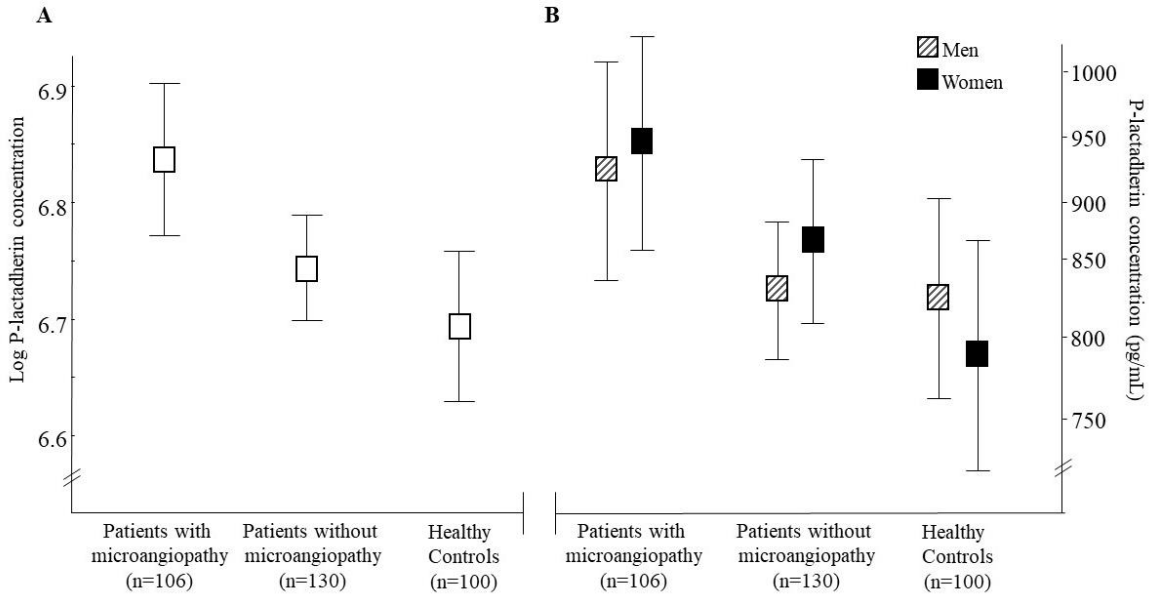


Figure 17. Plasma lactadherin levels in patients with type 1 diabetes and controls. The figure shows means and 95% C.I. of logged data on the left, with the corresponding P-lactadherin levels displayed on the right y-axis. **Figure A**: Patients with type 1 diabetes had significantly higher P-lactadherin levels than healthy controls ($p = 0.01$, ANOVA with contrast analysis of log-transformed data). Patients with microvascular complications had higher levels than patients without ($p = 0.02$). **Figure B**: P-lactadherin levels were higher in female patients with type 1 diabetes ($n = 107$) compared to healthy women ($n = 46$, $p = 0.01$), and significantly higher in female patients with microangiopathy compared to healthy controls ($p = 0.005$), whereas differences between female patients with and without microangiopathy as well as between female patients without microangiopathy and healthy women did not reach statistical significance. Men with type 1 diabetes and healthy men did not differ significantly in P-lactadherin, whereas male patients with microangiopathy tended to have higher lactadherin levels than male patients without microangiopathy ($p = 0.06$).

4.7 WOMEN WITH TYPE 1 DIABETES DISPLAY A DISPROPORTIONATELY PROTHROMBOTIC MV PHENOTYPE (PAPERS I AND II)

Among healthy controls, women had significantly lower lactadherin MFI, a measure of total PS expression on MVs in plasma, compared to men ($p < 0.001$), whereas this difference between men and women was not present among patients (Figure 18). Healthy women also had significantly lower total PMV levels and PS+ PMVs compared to healthy men ($p < 0.05$ for both, data not shown), despite of having higher plasma platelet concentrations ($196 \pm 42 \times 10^9$/L for men versus $221 \pm 38 \times 10^9$/L for women, $p = 0.01$). Men and women with type 1 diabetes, on the other hand, had similar PMV levels. After controlling for age, systolic blood pressure and use of tobacco, however, only the difference in total PMV levels between healthy men and women remained significant. For total MV levels as well as EMV subpopulations, no significant differences were found between men and women amongst either patients or controls.

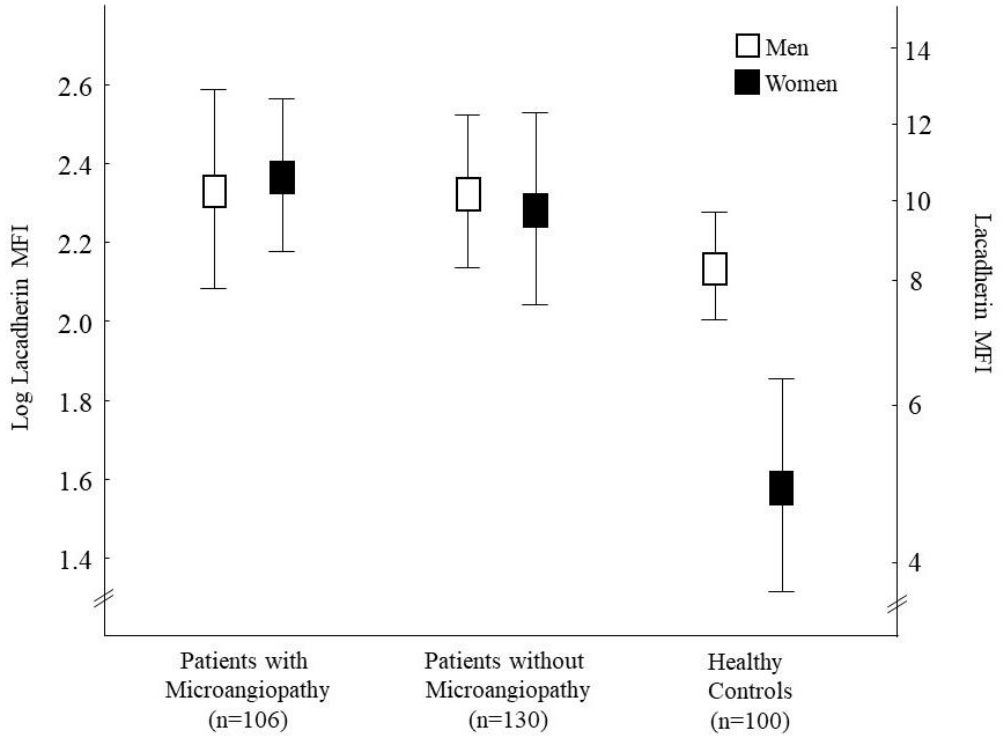


Figure 18. Phosphatidylserine expression on microvesicles in men and women with type 1 diabetes and controls.

Mean fluorescence intensity (MFI) of lactadherin is a measure of the total amount of phosphatidylserine (PS) expressed by MVs in plasma. The figure shows the means and 95% C.I. of logged data on the left y-axis, with corresponding unlogged data for lactadherin MFI on the right. Patients with type 1 diabetes mellitus (T1DM) as a group had significantly higher lactadherin MFI than healthy controls ($p < 0.001$), but with no difference between patients with and without microangiopathy. Women with type 1 diabetes had significantly higher lactadherin MFI than healthy women ($p < 0.001$), while men with T1DM and their healthy male counterparts did not differ significantly ($p = 0.13$). Healthy women had significantly lower lactadherin MFI than healthy men ($p < 0.001$), whereas men and women with T1DM had similar levels.

4.8 INFLUENCE OF PRE-ANALYTICAL HANDLING ON FLOW CYTOMETRY MEASUREMENTS OF MICROVESICLES (PAPER III)

4.8.1 Fresh samples analyzed using platelet gating

The number of phalloidin+ events in fresh samples did not differ between any of the centrifugation protocols compared to the reference protocol ($p > 0.05$ for all). CD42+ events/µL were significantly lower with the reference protocol compared to the other three protocols (protocol A/reference: 3 (2-5); protocol B: 22 (18-36); protocol C: 12 (10-15); protocol D 5 (4-11) CD42+ events/µL; $p < 0.001$ for B and C; $p = 0.02$ for D).

4.8.2 Frozen/thawed samples analyzed using MV gating

Following thawing, the A1 reference protocol where samples underwent no further re-centrifugation before MV analysis, resulted in significantly higher levels of phalloidin+ events/µL than those protocols that involved either low-speed or high-speed centrifugation of thawed samples prior to flow cytometry (i.e. protocols A2, A3, B2, B3, C2, C3, D2, D3; $p < 0.001$ for all, Figure 19). Those protocols that did not involve re-centrifugation following thawing did not differ significantly from the reference protocol, irrespective of which centrifugation protocol had been used prior to freezing (i.e. protocols B1, C1, D1; $p > 0.05$ for all, Figure 19). The number of total PS+ events/µL (regardless of specific cell type), did not differ between the A1 reference protocol and any of the other centrifugation protocols (data not shown). Total PMV levels were lower for those protocols that involved high-speed centrifugation after thawing (A2, B2, C2, D2) compared to the reference protocol A1 (Figure 20). For the protocols that involved low-speed centrifugation following thawing some significant differences were found, but there was no consistent pattern (see Figure 20). The effects of the different centrifugation protocols on PS+ PMVs were very similar to their effect on total PMV levels (data not shown).

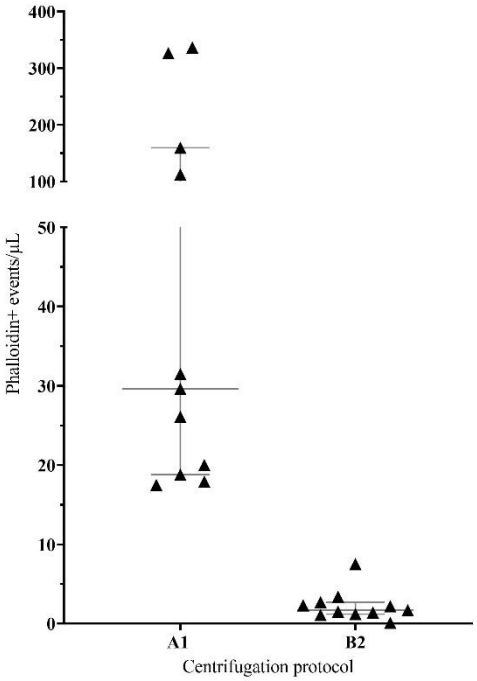
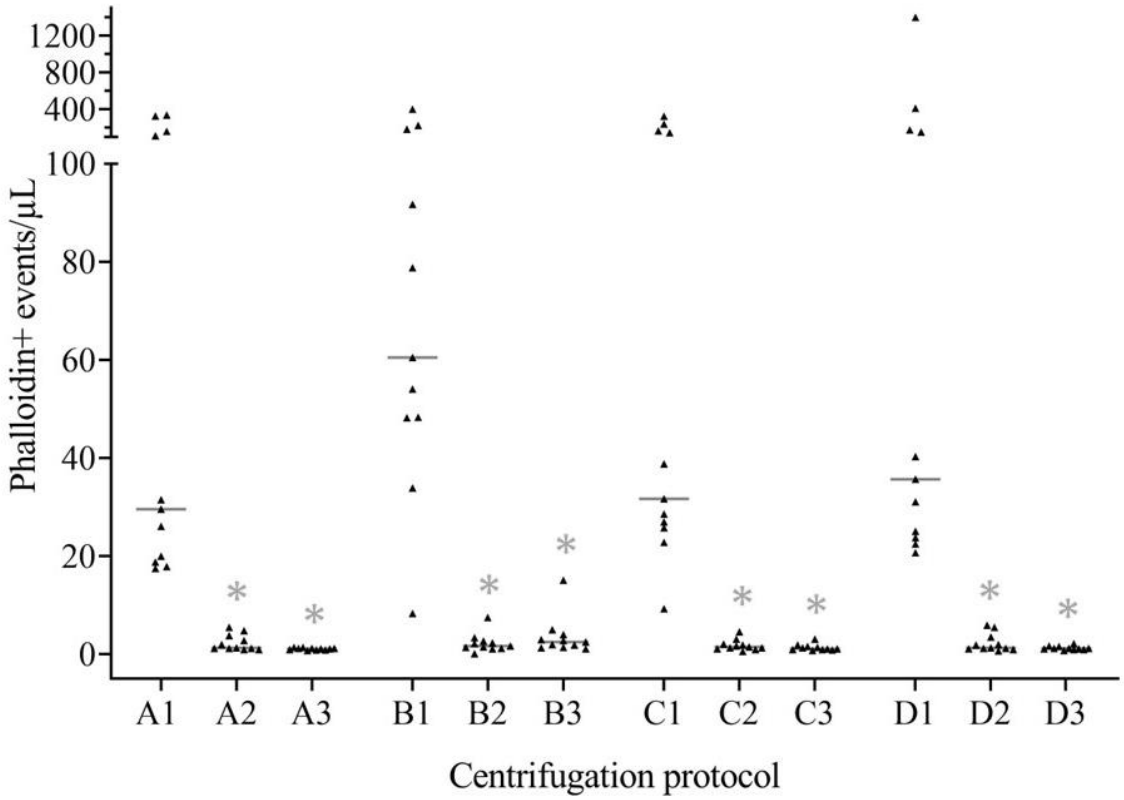


Figure 19. Effect of centrifugation on remaining cell fragments in thawed samples.

Below is a scatter plot of the number of phalloidin positive events per µL of frozen sample, isolated from healthy volunteers (n = 11) and handled according to different centrifugation protocols. Gray bars indicate median values. Asterix (*) denotes results that were significantly different from the reference protocol, A1 ($p < 0.001$ for all, Mann Whitney U).

For clarification, a direct comparison of the MISEV 2018 protocol (A1) versus our laboratory protocol (B2) is depicted on the left ($p < 0.001$). Figure shows medians and interquartile ranges.



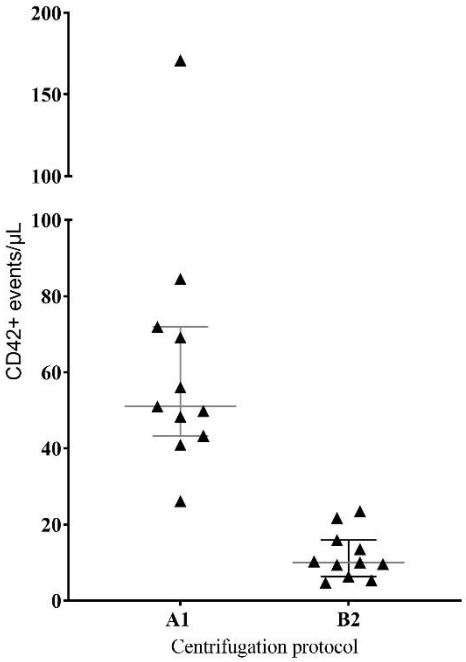
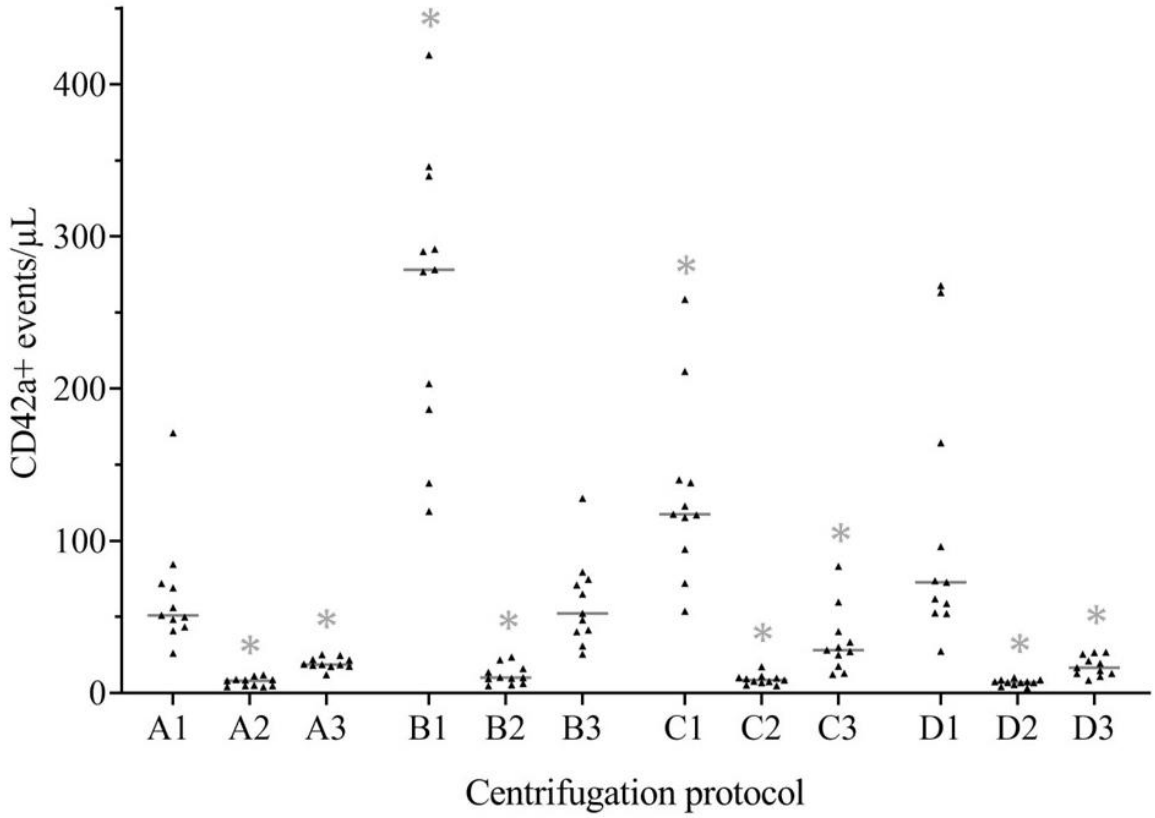


Figure 20. Effect of centrifugation on platelet microvesicles in thawed samples.

Below is a scatter plot of the number of platelet microvesicles (PMVs) per µL of frozen sample, drawn from eleven volunteers and handled according to different centrifugation protocols. Gray lines depict median values. Asterix (*) indicates results that were significantly different from the reference protocol, A1 ($p < 0.05$, Mann Whitney U). CD42 = glycoprotein IX, a platelet marker.

For clarification, a direct comparison of the MISEV 2018 protocol (A1) versus the laboratory protocol used in this thesis is shown on the left ($p < 0.05$). Figure shows median and interquartile range.



4.9 DISTURBANCES IN SKIN MICROVASCULAR FUNCTION CORRELATE WITH CLINICAL MICROANGIOPATHY (PAPER IV)

4.9.1 ACh and SNP iontophoresis in the forearm skin

Skin microvascular flux over time in response to iontophoresis with ACh (endothelium-dependent) and SNP (endothelium-independent) is shown in Figure 21. Compared to controls, patients with microangiopathy had a decreased microvascular response over time following induction with both ACh and SNP, whereas patients without microangiopathy did not differ significantly to healthy controls. Compared to patients without microangiopathy, patients with microangiopathy had reduced microvascular response to SNP but not to ACh (Figure 21, parts C and A). Patients were then divided into five different groups according to the severity of the clinical microangiopathy score described in Table 2. There was a gradual decrease in SNP-induced microvascular flux and a non-significant tendency towards a gradual decrease in ACh-induced microvascular flux as the clinical microangiopathy score increased (Figure 21, parts D and B).

Peak microvascular flux following iontophoresis with ACh and SNP were significantly reduced in both patient groups compared to controls ($p = 0.04$ and $p = 0.003$ respectively). Patients with microangiopathy also had reduced peak flux to SNP compared to patients without microangiopathy ($p = 0.006$) and showed a non-significant tendency towards a reduced peak response to ACh ($p = 0.12$).

There was a weak correlation between fP-glucose levels and peak microvascular flux in response to both ACh and SNP ($r = -0.29$ for ACh and $r = -0.25$ for SNP, $p < 0.05$ for both) as well as between BMI and peak flux ($r = -0.22$ for ACh and $r = -0.25$ for SNP, $p < 0.05$ for both). There were otherwise no significant correlations between either ACh- or SNP-dependent peak microvascular flux and HbA_{1C}, diabetes duration, blood pressure or lipid levels and no difference between patients who did or did not use tobacco.

4.9.2 Nailfold capillaroscopy and LDF of the big toe

Skin microcirculation was evaluated in the big toe using both nailfold videophotometric capillaroscopy to assess capillary blood flow, as well as laser Doppler fluxmetry (LDF) to assess total skin microcirculation. Unfortunately, results were only possible for about 2/3 of patients due to problems with skin thickness, skin damage, poor visualization, and insufficient LDF signals. Patients had higher basal capillary blood cell velocity (CBV) at rest compared to controls, whereas there was no difference in peak CBV. CBV ttp and porh also did not differ between patients and controls. Compared to patients without microangiopathy, patients with microangiopathy had lower CBV porh ($p = 0.03$), whereas there was no difference in the peak value. Baseline and peak LDF levels as well as skin temperature were higher in patients than controls ($p = 0.009$ and $p = 0.03$ for baseline and peak LDF and $p = 0.03$ for skin temperature),

but with no significant differences between the two patient groups. Peak LDF and peak CBV did not significantly correlate with each other.

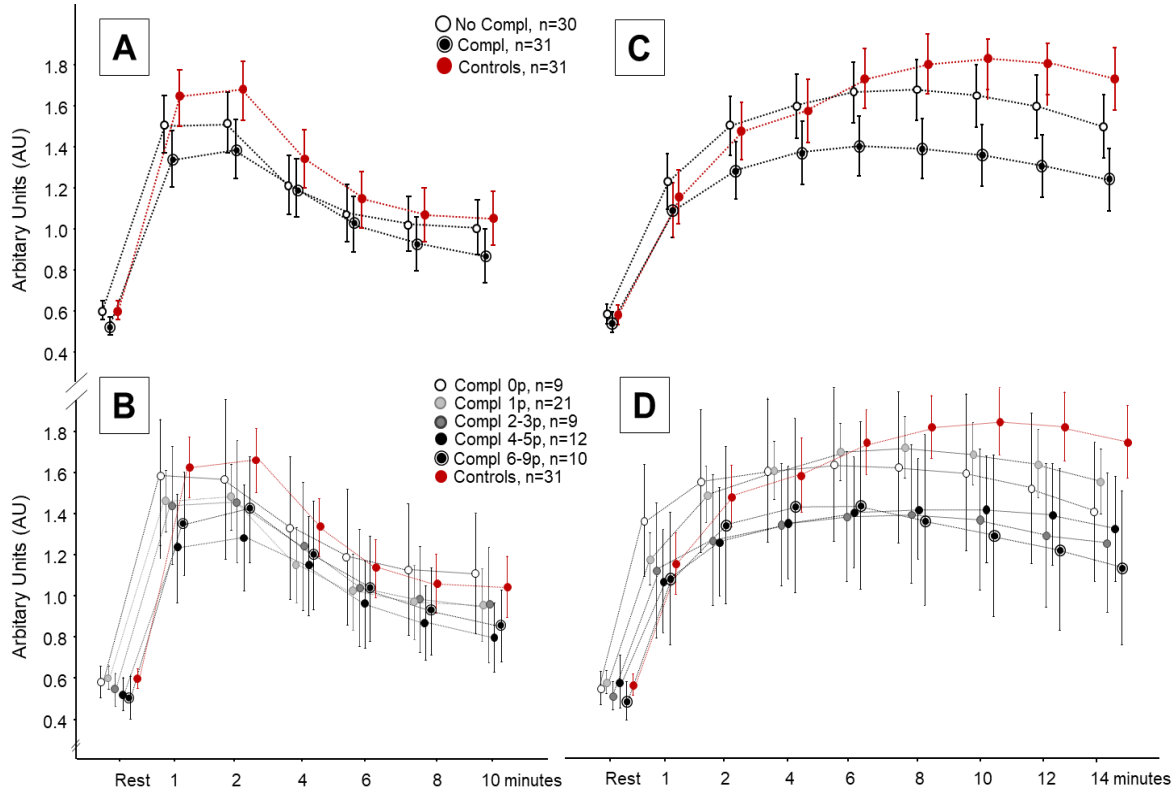


Figure 21. Changes in skin microvascular flux over time in response to iontophoresis with acetylcholine and sodium nitroprusside in patients with type 1 diabetes and controls.

The graph shows mean values with 95% C.I. for skin perfusion, expressed in arbitrary units (AU), before and following iontophoresis with acetylcholine (ACh), on the left, or sodium nitroprusside (SNP), to the right. Results for patients with no microvascular complications (white circles), patients with microvascular complications (black circles) and healthy controls (red circles) are shown in parts (A) and (C). Compared to controls, patients with microangiopathy had reduced responses to both ACh and SNP ($p = 0.03$ and $p < 0.001$ respectively, repeated measures ANOVA), whereas patients without microangiopathy did not differ significantly to healthy controls. Patients with microangiopathy had reduced microvascular response to SNP compared to patients without microangiopathy ($p = 0.01$, repeated measures ANOVA), whereas there was no significant difference between the two patient groups in their response to ACh. Responses of patients with different microangiopathy scores are shown in parts (B) and (D). Subgroup analysis indicated a nonsignificant gradual decrease in response to ACh and a significant gradual reduction in response to SNP ($p < 0.001$, repeated measures ANOVA) as the clinical microangiopathy score increased in severity.

4.10 CORRELATION BETWEEN PLASMA MICROVESICLE LEVELS AND SKIN MICROCIRCULATION (UNPUBLISHED DATA)

An explorative pilot study looking at the correlation between plasma MV levels and skin microvascular flux was carried out, including subjects from Papers I and II for whom both plasma MV levels and forearm microvascular response to iontophoresis with ACh and SNP had been measured. The pilot included a total of 17 patients with T1DM, of whom 13 patients had no clinical microangiopathy (apart from reversible simplex retinopathy) and 4 had established clinical microangiopathy, as well as 16 healthy controls. Among patients, there was a significant negative association between PS+ HMGB1+ PMVs as well as total HMGB1+ PMVs and peak response to ACh ($p = 0.03$, $r = -0.52$, $r^2 = 0.27$; $p = 0.02$, $r = -0.56$, $r^2 = 0.32$, respectively). There was also a tendency towards a negative association between PS+ HMGB1+ PMVs as well as total HMGB1+ PMVs and peak response to SNP ($p = 0.06$, $r = -0.46$, $r^2 = 0.21$; $p = 0.07$, $r = -0.44$, $r^2 = 0.20$, respectively).

A separate small pilot trial was conducted to analyze other proteins expressed on MVs that might be indicative of endothelial damage or hypercoagulability in T1DM. The pilot compared the five patients from Paper IV with the highest clinical microangiopathy scores as well as the most severe disturbances in their skin microvascular reactivity to five healthy controls. The expression of the following antigens on MVs was measured: tissue factor, tissue factor pathway inhibitor, eNOS, and endoglin. Looking at scatter plots comparing patients and controls, however, none of the markers showed any indication of being significantly different between the two groups (data not shown).

5 DISCUSSION

5.1 MV LEVELS ARE SIGNIFICANTLY ELEVATED IN PATIENTS WITH TYPE 1 DIABETES (PAPERS I AND II)

To the best of our knowledge, this thesis includes the single largest study to date looking specifically at MV levels in patients with type 1 diabetes in relation to clinical microangiopathy. We found significantly higher plasma levels of all subpopulations of measured MVs in patients with type 1 diabetes compared to matched healthy controls. This is in line with a large body of research in recent years that have implicated MVs as key mediators in a range of proinflammatory and prothrombotic disease conditions, including type 2 diabetes [105, 111, 112, 125, 129, 174], including diabetes [98, 102, 109, 122-124, 126-128, 175-177], although the majority of previous diabetes studies have been focused on type 2 diabetes.

Looking at MV subpopulations of different cellular origin, we were able to demonstrate that patients with type 1 diabetes have increases in MVs of both platelet as well as endothelial origin. The most abundant MVs in the circulation of both patients and controls were of platelet origin, which constituted 31% of the MVs measured (Table 5, aggregate analysis of patients and controls). This is in close agreement with the findings of Arraud et al., who studied MV levels in healthy subjects using electron microscopy and found 30% to be of platelet origin [178]. More recently, Berckman et al. measured plasma MV subpopulations in healthy controls using flow cytometry and showed that the platelet population constituted 52-56% of total MVs [179]. Discrepancies in exact percentages are likely explained by the fact that the studies have employed different cellular markers, measuring techniques, as well as flow cytometers with varying detection limits.

5.1.1 Patients display a disproportionate increase in PS-negative MVs

Somewhat unexpectedly, the largest contribution to the difference in total MV levels between patients and healthy controls came from a disproportionate increase in the level of PS-negative MVs. The proportion of total MV levels that were PS-positive in patients was only 31% (25 - 40%), compared to 44% (43 - 47%) for controls ($p < 0.001$). Most studies looking at MV levels in different patient populations have analyzed only total MVs or PS+ MVs of different cellular origins, while not including analysis of PS-negative MVs, the existence of which have been considered more controversial, and whose mechanisms of release and specific function remain unclear [108]. However, our findings are in line with results from Arraud et al., who employed cryo-transmission electron microscopy and receptor-specific gold labeling to visualize and describe the morphology of MVs in the plasma of healthy adults and were able to demonstrate that in fact only a minority of MVs exposed PS [178]. They speculated that such PS-negative MVs must be released through some cellular mechanism that preserves lipid membrane

asymmetry, and proposed cell fragmentation as a potential mechanism [178], although this has not yet been confirmed.

At this point it remains somewhat uncertain whether the high proportion of PS-negative MVs in patients with type 1 diabetes found in our study is a true reflection of the distribution of MV subpopulations in vivo. For instance, we cannot exclude the possibility that PS might be primarily expressed by smaller MVs with a diameter less than 0.2μm, which would be below the size detection limit of our flow cytometer.

There is a theoretical possibility that PS-negative MVs do in fact express PS, but at a concentration that is below the detectible threshold of our flow cytometer. However, we employed the use of lactadherin instead of the more commonly used Annexin V as a probe to detect exposure of PS on MVs. Studies have demonstrated that lactadherin binds to PS in a highly efficient calcium-independent manner [180, 181] and that it is able to detect extremely low levels of PS [182, 183]. Lactadherin is therefore recommended as the label of choice for accurate detection of procoagulant PS in MV studies [184]. As such, given the sensitivity of lactadherin for detection of PS, we believe that our results are in fact a true reflection of PS exposure on MVs and not a failure of detection.

Another explanation could be that PS exposure on certain MVs was masked by PS binding to circulating plasma proteins such as lactadherin. We therefore tested whether endogenous plasma lactadherin levels in patients could have interfered with our results but found no significant correlation between P-lactadherin and numbers or proportion of PS+ MVs. Of course, the existence of other interfering proteins found at higher concentrations in the plasma of patients cannot be completely excluded.

A physiologically plausible reason for the high proportion of PS-negative MS amongst patients might also be that PS+ MVs have a higher rate of turnover in the circulation due to the binding of PS to plasma lactadherin, which marks them for clearance by phagocytes [185]. It is worth noting that very little is known in general about the metabolism of MVs in vivo. Estimates of their half-time in the circulation are derived from the study of exogenous PS+ MVs following platelet transfusion and estimates vary between 10 minutes [186] and several hours [187]. The finding of a higher proportion of PS-negative MVs in patients with type 1 diabetes compared to healthy controls is thus consistent with a higher production of procoagulant PS+ MVs in patients coupled with higher rates of degradation of this MV subpopulation. In addition, studies have shown that endothelial cells themselves are active in internalizing PS+ MVs from the circulation [188]. If patients with type 1 diabetes have abnormally high levels of endothelial activation, as discussed below, it is possible that this serves to upregulate EC uptake of PS+ MVs, thus contributing to a higher proportion of PS-negative MVs remaining in the circulation.

On the other hand, several studies in the last decade have also found that the largest proportion of the increase in MV levels associated with different proinflammatory and prothrombotic conditions in facts consists of PS-negative MV subpopulations. Lundström et al. recently conducted a longitudinal study looking at the predictive utility of MVs following acute stroke

and showed that only PS-negative MV subpopulations were able to predict future cardiovascular events [189]. PS-negative MVs have also been shown to be disproportionately increased in SLE [190] and antiphospholipid syndrome [191]. The existence of PS-negative MVs in disease conditions clinically associated with hyperinflammation and hypercoagulability thus require further study.

5.1.2 Marked increase of endothelial MVs in type 1 diabetes

The most striking difference in MV levels between patients with type 1 diabetes and healthy controls was found for those of endothelial origin, where patients in our study had a median VE-cadherin+ EMV level of around 1100 counts/mL and an E-selectin+ EMV level of 3300 counts/mL, compared to only 39 and 41 counts/mL, respectively, for healthy controls ($p < 0.001$). E-selectin (CD62E) is a highly specific and sensitive biomarker of endothelial activation [192], which is upregulated in states of inflammation and plays a key role in the interaction of ECs with leukocytes in the circulation [193]. The elevated levels of E-selectin+ EMVs found in patients with type 1 diabetes in our study is thus indicative of a disturbed vascular homeostasis with pathological levels of endothelial activation, which is likely to further contribute to a disease state of chronic low-grade inflammation. Sustained endothelial activation is typically considered a first step towards more permanent endothelial dysfunction [192].

VE-cadherin (CD144), the other EC marker used in our study, is a critical component of the adherence junctions that connect ECs lining the luminal surface of blood vessels and thus help maintain the integrity of the vessel wall [194, 195]. As such, the dramatic increase in levels of circulating VE-cadherin+ EMVs in type 1 diabetes, is likely indicative of extensive underlying structural damage to the endothelium in patients. VE-cadherin+ EMVs signal increased vascular permeability, which would likely facilitate leukocyte extravasation from the blood vessel and thus promote vascular inflammation. It is also important to keep in mind that an intact endothelium is a critical regulator of hemostasis/thrombosis in the circulation. A damaged endothelium would likely have a decreased production of anticoagulant/fibrinolytic molecules, thus contributing towards a more procoagulant intravascular milieu. For comparison, a previous study analyzing EMV levels in antiphospholipid syndrome, a disease condition characterized by a marked propensity for thrombotic complications, demonstrated VE-cadherin levels of 6000 counts/mL on average [191].

Furthermore, given the important role of endothelial activation and dysfunction for macrovascular disease [192, 196], it is also quite possible that elevated EMV levels would increase the risk of heart disease and stroke. The exact role that EMVs may play as mediators in the development of cardiovascular disease in type 1 diabetes, and whether EMV levels at baseline can be used as clinically relevant biomarkers to be able to predict future disease, will have to be the explored in a future longitudinal study.

5.1.3 Moderately elevated PMV subpopulations in type 1 diabetes

PMVs and PS+ PMVs were also significantly higher in patients with type 1 diabetes compared to healthy controls, but the absolute difference between patients and controls was far less pronounced than for EMV levels, with a median PMV level of 9230 counts/mL for controls versus 11 345 for patients with type 1 diabetes ($p < 0.001$). Evidence of platelet hyperactivity in patients with type 1 diabetes without cardiovascular disease thus appears to be less distinct than disturbances in their endothelial homeostasis. Of course, it is important to keep in mind that patients with signs of macrovascular disease were actively excluded in this study.

It is also important to consider that in-vitro experiments have shown that the density of PS-expression on the surface of PMVs is much higher than is the case for activated platelets, such that the total procoagulant capacity of PMVs is up to 50-100 fold that of activated platelets [197]. Despite the relatively modest increase in PMVs and PS+ PMVs in patients with type 1 diabetes in terms of absolute numbers, this could therefore still mediate a potent shift towards a more procoagulant phenotype. Given the importance of platelet hyperactivity and thrombosis in macrovascular disease and the large number of studies implicating PMVs as active players in CVD [125], it would be of interest to conduct a follow-up study looking at the correlation between PMV levels, and in particular PS+ PMVs, and future development of cardiovascular disease in patients with type 1 diabetes.

5.1.4 Patients with type 1 diabetes have increased HMGB1+ MVs

Although previous studies have indicated that HMGB1 can be expressed on MVs in the circulation of healthy adults upon stimulation [198, 199], we have for the first time demonstrated a significant elevation of HMGB1+ MVs in the setting of type 1 diabetes. HMGB1+ PMV levels as well as HMGB1+ EMV levels (both VE-cadherin+ and E-selectin+) were all several orders of magnitude higher in patients compared to controls. Our findings imply that MVs can act as additional biological vectors for the transportation of proinflammatory HMGB1 in the circulation. Importantly, given the extensive research implicating HMGB1 in endothelial dysfunction, elevated levels of HMGB1+ MVs in type 1 diabetes might contribute to an increased risk of macrovascular disease.

Our results are in line with those of Marjanac and colleagues, who recently found significantly elevated levels of serum HMGB1 in 96 young patients with type 1 diabetes compared to healthy controls [200]. In contrast, an older study by Skrha et al. was only able to find an elevated level of serum HMGB1 in patients with type 2 diabetes but not type 1 diabetes compared to healthy controls [201]. The reason for this discrepancy is not clear, although it could be related to the use of different ELISA kits. A large subgroup analysis from the EURODIAB Prospective Diabetes Complications trial of type 1 diabetes looking at 463 patients with type 1 diabetes showed a positive correlation between serum HMGB1 levels and markers of low grade inflammation, endothelial dysfunction as well as the presence of albuminuria, whereas HMGB1 levels did not correlate with retinopathy or decreases in eGFR [202]. Why HMGB1

would correlate with albuminuria but not with decreases in kidney function is not completely clear, but a potential explanation is that HMGB1 and inflammation might be of particular importance for the initiation of podocyte injury in the early stages of diabetic nephropathy, whereas other mechanisms including fibrosis might be more critical for advancing chronic kidney disease at later stages [203].

5.1.5 Elevated HMGB1+ PMVs in type 1 diabetes are associated with a diminished vasodilatory response (unpublished data)

In a pilot study looking at the correlation between MV subpopulations and skin microcirculation in 17 patients and 16 controls, we found a significant negative correlation between both PS+ HMGB1+ PMVs and HMGB1+ PMVs and peak ACh response, and a trend towards a negative correlation to peak SNP response. This suggests that patients with higher levels of HMGB1+ PMVs may display a blunted capacity for vasodilation in the skin microcirculation. This finding supports a potential role of HMGB1+ PMVs as mediators of microvascular damage in type 1 diabetes, even though the significant differences in HMGB1+ PMV levels between patients with and without microangiopathy in the whole patient cohort did not remain once ANCOVA analysis had corrected for differences in baseline characteristics.

Our pilot study did not find any significant correlation between EMV subpopulations and response to iontophoresis with ACh or SNP. This is in line with our research also not revealing any clear association between elevated EMV levels and microvascular disease, which is what dysfunction of the skin microcirculation reflects. Previous work by Tehrani et al. looking at the effect of Atorvastatin on microvascular function in type 1 diabetes also did not find any correlation between endothelium-dependent microvascular flux and EMVs at baseline [204]. The lack of correlation between plasma EMVs and skin microvascular reactivity in such small samples may not be surprising as EMVs is an in-vitro marker in venous plasma samples, whereas microvascular flux is an in-vivo assessment of function in the microcirculation following provocation with pharmacological stimuli. The two might therefore not be directly related. In addition, the highly elevated plasma EMV levels found in all patients with type 1 diabetes in Paper II, regardless of their microangiopathy status, may also suggest that plasma EMVs more accurately reflect the large blood vessels rather than the microcirculation.

5.1.6 MVs did not correlate significantly with clinical characteristics

Surprisingly, MV subpopulations in our study did not correlate strongly with any of the traditional risk factors for either microvascular or macrovascular disease, including metabolic control, with almost all of the associations having a r-value below 0.3. One explanation might be that we only measured P-glucose at one time-point (in the fasting state), which would have missed recent fluctuations in blood sugar which could theoretically have influenced MV levels,

given that both hyperglycemia as well as hypoglycemia have been shown to induce increased MV formation [120, 205]. It is also important to keep in mind that HbA_{1C} is a somewhat crude estimate of metabolic control, which does not give an indication of glucose variability.

Of note, in a recent study of Annexin V+ (PS+) PMVs and EMVs in pregnant women with type 1 diabetes randomized to either continuous glucose monitoring or self-assessed glucose monitoring, there was also no consistent relationship between EMVs and either mean glucose levels, HbA_{1C}, or measures of glucose variability [206]. The study found a weak negative correlation between Annexin V+ PMVs and time with glucose above target range as well as glucose standard deviation. Despite of improvements in glycemic control in the group randomized to continuous glucose monitoring, however, the study was unable to detect any differences in MV levels between the two groups. The authors speculated that there are probably multiple factors responsible for MV formation, such that any clear association to a single factor might be difficult to detect [206].

5.2 NO CLEAR CORRELATION BETWEEN MV SUBPOPULATIONS AND CLINICAL MICROANGIOPATHY (PAPER I AND II)

We compared plasma concentrations of total circulating MVs, PMVs, and EMVs as well as MV expression of PS and HMGB1 between patients with and without clinical microangiopathy. Contrary to our original hypothesis, we found no clear evidence of a correlation between any of the MV subpopulations and the presence of clinical microangiopathy.

The largest difference between patients with and without microangiopathy was found for HMGB1+ PMVs, where patients with microvascular complications had 89% higher median levels compared to patients without (median 648 (165 - 1048) vs 343 (101 - 858)). This difference was statistically significant in ANOVA analysis ($p = 0.04$, Figure 15). However, in ANCOVA analysis controlling for differences in baseline characteristics between the two patient groups (age, systolic blood pressure, diabetes duration, eGFR, fP-glucose, HbA_{1C} and the use of ACE-i, ARBs, statins, or insulin treatment modality), this difference did not remain ($p = 0.42$). Since our study was not specifically powered to detect differences in HMGB1+ MV subpopulations, type 2 error remains a possibility.

The lack of correlation between MV levels and microangiopathy was somewhat surprising given a number of convincing in-vitro and animal studies demonstrating a clear pathophysiological role for MVs in the promotion of thrombus formation, inflammation, oxidative stress, endothelial microvascular dysfunction and platelet-endothelial crosstalk, particularly in the setting of hyperglycemia [117, 120, 132, 134, 188, 207, 208].

On the other hand, human studies looking at the link between clinical microangiopathy in type 1 diabetes and MV levels have been somewhat conflicting. Sabatier et al. conducted the first study looking at MV levels in 24 patients with type 1 diabetes [135]. They found no relation

between PMV levels and microangiopathy but a trend towards higher CD51+ (Integrin alpha 5) EMV levels in patients with microvascular complications compared to those without, although the study was too small to draw any definitive statistical conclusions [135]. Salem et al., meanwhile, found higher CD41+ PMV levels in patients with microvascular complications than those without in a cohort of 80 children with type 1 diabetes (mean age 10) [136]. They concluded that PMV levels could be used as biomarkers of microangiopathy, and also hypothesized that PMVs might link microangiopathy with macrovascular disease, given a positive correlation between PMV levels and carotid intima media thickness [136]. However, it is very difficult to compare their results with our study since they analyzed PMV levels in whole blood rather than plasma, which is not typical. Their study population was also significantly younger, had worse metabolic control, higher rates of diabetic nephropathy (40% vs 12% in our study) and lower rates of retinopathy (7.5% vs 36% in our study) compared to our study population.

Several other small studies have also shown elevated levels of different MV subpopulations in patients with type 1 diabetes compared to healthy controls [209-211], however, they have not looked specifically at the link between MV levels and clinical microangiopathy. In contrast, a recently published longitudinal study by Bratseth et al. comparing Annexin V+ (PS+) MVs in 40 children with type 1 diabetes (median age 14, median diabetes duration 5 years, median HbA_{1C} 69 mmol/mol) versus 40 healthy controls actually found no difference in plasma MV levels between the two groups at baseline [212]. The authors speculated that the young age and relatively short diabetes duration of the study population, as well as the absence of microvascular complications, might be a potential explanation. Surprisingly, when MV levels were measured again after a 5-year follow-up period, patients had significantly lower Annexin V+ MV levels than controls, despite of further deterioration in their metabolic control (median HbA_{1C} 79 mmol/mol) as well as significantly higher albumin excretion ratio. After controlling for covariates, however, MV levels in patients and controls were again similar. The authors proposed that frequent administration of insulin might exert protective effects on the endothelium lining, despite of not achieving target glucose levels [212]. The results of our study, with strikingly high levels of EMV levels in patients regardless of microangiopathy, does not support the theory that insulin administration is sufficient to protect the endothelium from pathological activation in the setting of diabetes. Importantly, Bratseth and colleagues did not analyze PS-negative MV levels, which was the MV population that increased the most in patients in our study and which differed the most compared to controls.

It is worth noting that in our study, even patients with microangiopathy were still relatively healthy, frequently only showing signs of clinical microangiopathy in one organ system. This is probably due to macrovascular disease being an exclusion-criteria in the study, since these two conditions often go hand in hand. For EMV levels, which were markedly increased in all patients compared to controls, it is therefore possible that the background endothelial activation and dysfunction in type 1 diabetes was so high that it masked any minor differences between patients with or without microvascular complications, which the study may have been underpowered to detect. However, even if a larger study might have reached statistical

significance, given how small the absolute differences in EMV levels were between the two patient groups, it is unlikely that EMV levels could be applied as a clinically meaningful biomarker for microangiopathy at the individual level.

It is possible that the presence of other comorbidities influencing MV levels in adult patients with T1DM, such as obesity, insulin resistance, hypertension, smoking and other factors, makes it difficult to use MVs as biomarkers in T1DM except for at a very young age. Although we tried to control for these factors in the statistical analysis using ANCOVA, this may not have been sufficient, and there might also have been other confounders unaccounted for in our calculations.

Another possible explanation for the lack of a significant difference in MV levels between patients with and without microvascular complications might lie in the complex nature and diverse biological functions of MVs in and of themselves. Studies in recent years have demonstrated that the exact role of MVs in driving inflammation and thrombosis is far more nuanced than what was previously believed to be the case. Whereas earlier studies focused on elucidating the procoagulant properties of MVs, experiments in recent years have demonstrated that MVs can in fact have the opposite effect under specific conditions [112, 213]. For instance, Berckman et al. showed that MVs in healthy humans can stimulate plasmin formation, thereby promoting fibrinolysis [179]. Similarly, certain MV subpopulations may exhibit antioxidant as well as immunomodulatory properties under specific conditions [105]. MV molecular cargo and surface signatures depend on the surrounding physiological or pathophysiological conditions and are regulated by the specific trigger for their release [105]. It has thus become apparent that the types of MV subpopulations released are highly heterogeneous and their biological actions can act in opposing directions. It is therefore plausible that total MV, EMV and PMV levels might be too unspecific to be used as biomarkers of clinical microangiopathy, since these parameters might mask more subtle and dynamic shifts in the proportions of specific subpopulations of MVs. There might also be other surface antigens or key miRNA sequences contained inside the MVs that are of greater importance for promotion of microangiopathy in type 1 diabetes than PS or HMGB1, and which are yet to be identified.

Finally, although the microvasculature in the body makes up the largest proportion of the total circulation by far, it is important to keep in mind that assessment of plasma MVs in blood samples taken from an antecubital vein does not allow for assessment of which part of the circulation they originated from. In contrast, studies of skin microcirculation allow for isolated assessment of the microcirculation, the part of the vascular system involved in microangiopathy. Given that endothelial dysfunction is a shared mechanism contributing to both microvascular and macrovascular disease, it would be interesting to study the association between EMV levels and macrovascular disease in type 1 diabetes in a longitudinal study.

5.3 WOMEN WITH TYPE 1 DIABETES HAVE A DISPROPORTIONATELY PROCOAGULANT MICROVESICLE PHENOTYPE (PAPERS I AND II)

Our study found that healthy women had significantly lower lactadherin MFI ($p < 0.001$, Figure 18), total PMV levels and PS+ PMV levels ($p < 0.05$ for both) compared to male controls, although the difference in PS+ PMV levels did not remain significant after controlling for covariates. In contrast, there were no differences in PMV levels or lactadherin MFI between men and women with type 1 diabetes. Lactadherin MFI is a measure of the total PS exposure on all MVs in plasma. As such, these results indicate a disproportionate increase in platelet activation and procoagulant potential in the plasma of women with type 1 diabetes. This is line with earlier findings by Tehrani et al. showing a tighter fibrin network in young women with type 1 diabetes compared to young male patients [47]. Such hemostatic disturbances in women with type 1 diabetes might help explain why female patients experience a relatively larger increase in cardiovascular risk than male patients [16, 42, 43].

5.4 MICROVESICLES FROM PATIENTS DISPLAY A MORE REACTIVE PROCOAGULANT PHENOTYPE, BUT NO DIFFERENCE IN TOTAL THROMBIN FORMATION (PAPER I)

Our study included functional assessment of the differential ability of MVs isolated from patients with T1DM versus healthy controls to promote thrombin generation in-vitro using a modified version of CAT, without the addition of any other triggering reagent. MVs isolated from patients resulted in significantly shorter lag time ($p = 0.01$) as well as a shorter time to peak ($p = 0.006$), which indicates a more reactive procoagulant MV phenotype in patients (Figure 16). On the other hand, the peak levels of thrombin and the ETP did not differ significantly between patients and controls, meaning that the total amount of thrombin generated by MVs was ultimately the same (Figure 16). Whether or not the propensity for MVs isolated from patients to induce faster clot formation has any real clinical significance is uncertain.

Importantly, the addition of lactadherin led to almost complete absence of MV-induced thrombin generation in our study. This is an indication of the important mechanistic role that PS-expression plays in the ability of MVs to propagate coagulation by acting as a catalytic surface for the formation of the tenase and pro-thrombinase complexes [115]. Given that most of the increase in MV levels amongst patients with T1DM consisted of PS-negative MVs, this might help explain why patients ultimately did not differ from healthy controls in the total amount of thrombin generated. We were also not able to detect any differences in CAT parameters between patients with and without microvascular complications. This is consistent with the fact that patients with and without microangiopathy had similar levels of PS+ MVs.

5.5 P-LACTADHERIN IS INCREASED IN TYPE 1 DIABETES AND CORRELATES WITH CLINICAL MICROANGIOPATHY (PAPER I)

We have for the first time demonstrated increased plasma lactadherin levels in patients with type 1 diabetes, with a correlation to the presence of microvascular complications. Lactadherin is a small soluble glycoprotein involved in the clearance of apoptotic cells, which has increasingly been recognized as playing an important role in the regulation of inflammation, hemostasis, and vascular remodeling [214, 215]. Lactadherin is expressed by endothelial cells but also in the surrounding vessel wall [216]. It is released into the circulation from macrophages and immature dendritic cells, where it plays a crucial role in efferocytosis, the clearance of activated or apoptotic cells or MVs from the circulation [217, 218]. It acts as an opsonin or bridge between cells by binding integrins on phagocytes via its C-terminus motif and attaching to PS on cells marked for apoptosis via its N-terminus domain [185]. Furthermore, it has been shown that the clearance of PS+ cells is stereospecific and particularly effective for more highly curved particles, meaning that clearance of PS+ MVs via lactadherin is particularly expedient [219, 220]. The higher lactadherin levels found in patients with type 1 diabetes in our study could reflect higher turnover of both cells and MVs in the circulation of patients. High cell turnover may result from pervasive inflammation, abnormal cell activation and vessel wall damage, especially amongst patients with established microangiopathy.

Research in SLE has demonstrated that there appears to be an optimal level of lactadherin in the blood for efficient phagocytosis, such that both low and high levels are correlated with increased disease severity [221]. On the one hand, effective phagocytosis of damaged cells is crucial to prevent secondary necrosis and accumulation of cellular debris with the potential to trigger inflammation [222]. To this effect, lactadherin has been shown to protect against tissue fibrosis by mediating the clearance of defective collagen [223]. On the other hand, pathological levels of apoptosis can be detrimental. For example, a study of human umbilical vein endothelial cells under hyperglycemic conditions identified lactadherin induced by AGEs as a key mediator in the promotion of apoptosis of ECs [224].

The role of lactadherin as an important regulator of hemostasis is also increasingly being recognized [214]. Gao et al. have previously demonstrated that in the setting of uremic hypercoagulability in chronic kidney disease patients, the addition of lactadherin to block PS-exposure on endothelial cells, peripheral blood cells and MVs was able to decrease up to 80% of prothrombinase and tenase formation in vitro, thus decreasing thrombin and fibrin formation [225]. It is thus possible that elevated lactadherin levels in the circulation of patients with type 1 diabetes acts as a protective mechanism to prevent excess coagulation. As such, the significance of increased plasma lactadherin levels in patients with type 1 diabetes is not completely clear at this point, as it could potentially have either beneficial or harmful biological effects.

Our research is in concordance with a previous study by Cheng et al. showing that patients with type 2 diabetes have increased levels of serum lactadherin compared to healthy controls, in particular patients with microvascular complications or decreased pulse-wave velocity [226].

In contrast, a more recent study by Sun and colleagues found lower levels of lactadherin in patients with type 2 diabetes with microvascular complications compared to those without complications [227]. It is worth noting that both these studies are considerably smaller than the one included in this thesis, and Sun et al. did not enroll a control group without diabetes, which makes it more difficult to interpret their results. Li et al. recently replicated the finding that patients with type 2 diabetes have elevated levels of lactadherin, but also found that lactadherin levels were suppressed in obese subjects, such that only non-obese diabetic patients had increased lactadherin levels [228]. In our study, however, there was no correlation between lactadherin and BMI.

5.6 DEVELOPMENT OF A SIMPLE LABORATORY PROTOCOL FOR USE OF MICROVESICLES AS CLINICAL BIOMARKERS (PAPER III)

The flow cytometry measurements of MVs analyzed in Paper I and II were carried out prior to the publication of the latest methodological guidelines concerning flow cytometry analysis of MVs [103]. The MISEV 2018 guidelines endorses the protocol recommended by the International Society on Thrombosis and Haemostasis to use two consecutive rounds of centrifugation at 2500g for 15 minutes in order achieve close to platelet-free plasma prior to freezing [163, 164], thus decreasing the likelihood of ex-vivo release of MVs during storage [165, 166]. After thawing, MISEV 2018 recommends direct flow cytometry analysis, without prior re-centrifugation. Our group consequently designed a laboratory experiment to study the effect of different centrifugation protocols on MV parameters. As outlined in detail in the Methods, we compared the MISEV 2018 protocol to different laboratory protocols that used a single centrifugation step prior to freezing, following by re-centrifugation of thawed samples prior to analysis (Figure 9). Not surprisingly, the MISEV 2018 protocol using double centrifugation of fresh samples resulted in significantly lower levels of remaining platelets before freezing ($p < 0.001$ compared to all other protocols), indicating that samples were closer to being truly platelet-free, which should theoretically decrease the risk of ex-vivo release of MVs. However, as shown by measurements of phalloidin [167], the omission of the re-centrifugation step prior to MV analysis of thawed samples resulted in significantly higher levels of remaining cell fragments, increasing the likelihood of artefacts and false positive events in the subsequent MV analysis (Figure 19). On the other hand, using high-speed centrifugation after thawing, as was our laboratory practice when conducting flow cytometry analysis for Papers I and II, resulted in significantly lower PMV and PS+ PMV levels than the MISEV protocol (Figure 20). This indicates that some of the particles of interest were likely removed in the process of attempting to remove cell fragments. In contrast, the B3 protocol which used a single round of low-speed centrifugation both prior to freezing and after thawing, did not result in lower PMV levels compared to the MISEV 2018 protocol.

Importantly, despite of numerous studies in the last decades pointing to the important role of MVs as mediators in a range of proinflammatory and prothrombotic chronic diseases, including diabetes and CVD [98, 105, 110, 122, 125-128, 229], this knowledge has so far failed to be

implemented into clinical practice. The MISEV 2018 guidelines [103], although admirable in their ambition to standardize experimental conditions across different laboratories, fail to take into account the practical difficulties of using double-centrifugation prior to freezing in larger clinical trials or routine medical practice. It is also important to remember that rigorous standardization of sample handling within any one MV study, such that plasma samples of patients and controls are handled carefully according to an identical protocol, should allow for valid comparisons between subjects [230].

As such, considering that both the MISEV protocol and the other centrifugation protocols all have benefits as well as drawbacks, we propose that a carefully standardized centrifugation protocol using a single round of centrifugation prior to freezing, following by low-speed centrifugation after thawing to remove remaining cell debris and remediate samples, could be a simple and pragmatic method for preparing samples for MV analysis in larger clinical trials. This approach would also be feasible to one day use in standard clinical practice.

5.7 SKIN MICROCIRCULATION CORRELATES WITH CLINICAL MICROANGIOPATHY (PAPER IV)

Our study of skin microcirculation in 61 patients with type 1 diabetes and microvascular disease of varying severity and 31 matched controls showed a clear association between the degree of clinical microangiopathy and the level of skin microvascular disturbances. Our findings suggest that microangiopathy in type 1 diabetes is systemic condition, which affects microvascular beds in many different parts of the body. Our results also add to the growing body of evidence indicating that the skin microcirculation can be used as an easily accessible model for the study of microvascular disease.

Patients with microvascular complications showed a decreased vasodilatory response over time to both ACh and SNP compared to healthy controls. They also displayed a significantly more blunted reaction to SNP compared to patients without complications (Figure 21). Peak responses to ACh and SNP were significantly lower in patients with and without complications compared to controls, whereas the difference between patient groups was only significant for SNP. Subgroup analysis comparing patients with different clinical microangiopathy scores strengthened our findings, since the severity of clinical microangiopathy was mirrored by the degree of disturbance that patients exhibited in skin microvascular response, although because of the small size of the subgroups this only reached statistical significance for SNP (Figure 21).

ACh exerts its action by binding to receptors on ECs and mediating the release of NO, prostaglandins, and endothelial-derived hyperpolarization factor, which indirectly cause relaxation of smooth muscle cells via paracrine signaling. SNP, on the other hand, bypasses the endothelium and directly activates intracellular NO-sensitive guanylyl cyclase, leading to smooth muscle relaxation [158]. If there were an isolated reduction in response to ACh, this would thus point to endothelium-dependent impairment. In our case, however, both responses to ACh and SNP were diminished. This is consistent with smooth muscle cell involvement but

doesn´t help us clearly differentiate the contribution of the endothelium, given that the vasodilatory effect of the endothelium on the microvasculature is mediated through the action of smooth muscle cells. Given the distinct elevation of E-selectin+ and VE-cadherin+ EMVs in patients with type 1 diabetes found in Paper II of this thesis, however, we believe it likely that endothelial dysfunction was also involved. It has been proposed that skin microvascular dysfunction in T1DM is multifactorial, caused by a combination of functional impairment involving endothelial cells and decreased NO bioavailability, as well as structural changes to the vessel wall, including the smooth muscle cells [40, 148]. In our study, differences between patients with and without complications were only significant for SNP, perhaps indicating that pathological changes affecting endothelial-independent pathways may play a more important role in skin microvascular dysfunction.

Our findings are in agreement with a growing number of studies that have demonstrated decreased skin microvascular responses following pharmacological provocation with iontophoresis in type 1 diabetes [148, 149, 151-154, 231], although only a few studies to date have compared patients with and without clinical microangiopathy. Rathsman et al. found an association between presence of severe retinopathy and having a decreased response to ACh iontophoresis in type 1 diabetes [154]. Nguyen et al., on the other hand, found that type 1 and type 2 diabetes patients with diabetic retinopathy had reduced microvascular responses to ACh and SNP compared to patients without retinopathy, although this did not reach statistical significance when looking at patients with type 1 diabetes in isolation [231].

Whether T1DM is primarily associated with a disturbance in endothelium-dependent or endothelium-independent microvascular reactivity, or both, has varied in the different studies, as did the correlation between skin microvascular reactivity and clinical parameters such as metabolic control and diabetes duration [148-153]. Discrepancies between studies could be caused by several factors including a lack of method standardization (different concentrations of iontophoresis drugs, single versus repeated doses, fasting versus not fasting subjects, use of different sites for testing including the forearm, hand, and foot), as well as the considerable heterogeneity of the patient groups involved in the studies (age, metabolic control, and diabetes duration).

Interestingly, our study showed that a number of patients with type 1 diabetes and median disease duration of over 30 years, had developed no microvascular complications and also had skin microcirculation responses similar to healthy controls, meaning that they had somehow been protected from the typical vascular damage commonly caused by long-standing hyperglycemia. Patients without microangiopathy in our study had significantly better metabolic control than patients with complications (mean HbA_{1C} 55 ± 8 versus 66 ± 13 mmol/mol, respectively), but they still clearly experienced supra-physiological levels of blood glucose compared to those found in healthy controls ($p < 0.001$, Table 4). Despite of the presence of hyperglycemia and its pro-oxidative and proinflammatory effects, their skin microvascular function had remained relatively intact. Furthermore, as clearly demonstrated by results from Papers I and II, all patients with type 1 diabetes, regardless of the presence of

microangiopathy, appear to have significantly elevated levels of MV levels, especially of endothelial origin. Although MV levels were not measured in the study population of Paper IV, evidence would thus suggest that the patients in the study most likely had significantly higher MV levels than healthy controls. Even so, their microvascular function remained relatively unaffected. This again speaks to the existence of some yet to be fully elucidated protective factors, preventing vascular damage in select patients with type 1 diabetes [48], which is line with earlier findings from the Joslin Diabetes Center 50-Year Medalist Study [49]. Although recent progress has been made in trying to elucidate the biological mechanisms behind this, including looking at polymorphisms in genes coding for inflammatory proteins [52] and variations in liver biosynthesis of nucleotides related to insulin resistance [53], more work is still needed before this knowledge can be translated into clinical practice.

Nailfold videophotometric capillaroscopy as well as single-point LDF of the big toe indicated a higher basal skin blood flow in patients compared to controls ($p = 0.03$ and $p = 0.009$, respectively). This was further corroborated by patients having higher basal skin temperature at the toe nailfold ($p = 0.03$). Our findings agree with previous research indicating that the early stages of microangiopathy in diabetes are characterized by a maladaptive increase in microvascular flow, causing increased capillary pressure and contributing to basement membrane thickening and tissue fibrosis, ultimately leading to reduced vasodilatory responses and a diminished capacity for autoregulation of blood flow [147, 232]. This is similar to the changes observed in the glomeruli in the setting of diabetic nephropathy, which precedes development of albuminuria [54]. In contrast, Sorelli et al, recently studied nailfold LDF at the big toe in 47 patients with T1DM and found decreased cutaneous perfusion at baseline in patients compared to controls [233]. The reason for this difference is not clear but could perhaps be related to differences in patient characteristics. Presence of microangiopathy or neuropathy amongst patients in their study was not detailed.

LDF peak was increased in patients compared to controls ($p = 0.03$), whereas patients with and without microangiopathy did not differ. However, results for LDF of the big toe in our study must be interpreted with caution, since unfortunately measurements could only be completed for about 2/3 of patients.

Evidence from nailfold capillaroscopy studies that have employed visual rating of structural microvascular abnormalities observed by nailfold capillaroscopy, including avascular areas, microaneurysms, capillary enlargements, excessive angiogenesis and microhemorrhages [234], have demonstrated a clear link between more severe microvascular changes and the presence of microangiopathy in patients with type 1 diabetes [156, 235-237]. The downside of visual grading of structural changes is that they contain an element of subjectivity, whereas videophotometric capillaroscopy assessing capillary blood flow employed in our laboratory allows for more quantitative assessment. In our study, CBV peak was not significantly different between patients and controls or between the two patient groups. Unfortunately, the large drop-out in patients due to insufficient quality of data in the nailfold videophotometric capillaroscopy does not allow for proper interpretation of results.

We piloted the use of a simple clinical microangiopathy scoring system that allowed for comparison between patients with microangiopathy across different vascular beds. We believe that this can be a useful tool in future research studies, and it may also aid in formalized risk-assessment of patients in the clinic.

5.8 STRENGHTS

The studies included in this thesis have several strengths. The patient population included in Papers I/II was large, and the two papers together constitute the largest study to date specifically looking at MVs in relation to microangiopathy in type 1 diabetes. Patient characteristics were described in detail, and the study included a carefully selected control group matched for sex, age, and BMI. All plasma samples analyzed in the study were processed carefully according to a standardized protocol, avoiding any differences in pre-analytical handling between patients and controls and ensuring that any potential sources of laboratory error would be evenly distributed between the two groups. Plasma samples for MV analysis were drawn under fasting conditions, which is important given that food intake has been shown to increase levels of procoagulant MVs [238]. Taking samples following over-night fast should also have minimized the presence of chylomicrons that could be co-localized with MVs and mistaken for PS-negative MVs in flow cytometry analysis. Plasma samples were frozen and thawed only once before analysis, thus minimizing the effect of freezing on MV numbers or PS exposure [163]. Furthermore, we used phalloidin to control for remaining cell-fragments to ensure sample quality [167], with only samples with <10% phalloidin included in the analysis. Lactadherin was used to detect procoagulant PS, which has the advantage of binding in a calcium-independent manner and has also been shown to be a more sensitive probe than the more commonly used Annexin V [180-182, 239]. Furthermore, lactadherin has been shown to have a significantly lower tendency for mistakenly binding to PS expressed by lipoproteins compared to Annexin V [240].

For Paper IV, the study was designed specifically to be able to detect differences in ACh-induced peak flux between patients with and without microangiopathy, which has not been the case for previous studies looking at skin microcirculation in type 1 diabetes. The study population was thoroughly described in terms of clinical characteristics and included patients with long disease duration with and without microangiopathy, as well as a meticulously matched control group. We piloted the use of a simple clinical microangiopathy score, which was beneficial since it allowed for comparison of patients with varying degrees of microangiopathy involving different vascular beds. In contrast, previous studies looking at the link between skin microcirculation have only looked at retinopathy in isolation [154, 231]. Finally, we used laser Doppler imaging to assess microcirculatory responses following iontophoresis, which has been shown to be a more reliable method with greater reproducibility than single-point LDF [241-243].

5.9 LIMITATIONS

For the clinical interpretation of Papers I/II as well as IV, it is important to keep in mind that these are cross-sectional in nature, meaning that the causality of any significant relationships between variables cannot be determined for certain.

The flow cytometry studies in this thesis were all carried out prior to the publication of the MISEV 2018 methodological guidelines for the study of extracellular vesicles [103] and the MIFlowCyt-EV standard for reporting in EV studies [244]. As such, our centrifugation protocol is not in accordance with the MISEV recommendations. However, we have employed a carefully standardized protocol that was identical across groups, meaning that any unintended error should have been carried over equally and the effect of pre-analytical handling should have been minimized [230]. Importantly, as demonstrated in Paper III, there are also drawbacks to the protocol endorsed by MISEV, including a higher degree of remaining cell fragments, increasing the risk of false positives. Given the extreme difficulty of accurately assessing absolute MV numbers in any study [178], we would argue that within-study standardization remains the single most important remedy to allow for comparisons between subjects.

The lower detection limit of our flow cytometer was around 0.2 - 0.3 µm, meaning that we were unable to measure the smallest MVs. We also did not specifically control for unintended co-isolation of lipoproteins, which might have been mistakenly identified as MVs. However, collecting plasma samples under fasting conditions should have minimized the existence of chylomicrons, and lactadherin has a very low affinity for binding to PS on lipoproteins [240].

Another potential issue in MV studies is the possibility of swarm detection, which is when MVs cluster together such that the emitted signal detected by the flow cytometer is mistaken as originating from a single particle [245]. Although we did not specifically dilute samples for this purpose, the addition of antibodies as well as CellFix should have diluted the samples sufficiently to reduce this risk [179]. The use of polystyrene beads to try to set the gate for EVs is oversimplistic due to differences in the refractive index (RI), meaning that they can differ significantly in diameter [178]. However, our use of unmarked MV samples to control the setting of the gates should have improved accuracy. Future improvements might include the use of hollow organosilica beads with an RI more similar to that of MVs [246], as well as the use of computer algorithms to correct for different scatter-diameter relationships [247, 248].

For Paper IV, a potential confounder is the higher prevalence of hypertension and use of antihypertensives amongst patients with microangiopathy, although this has been shown to not significantly affect skin microcirculation [249, 250]. As mentioned, nailfold capillaroscopy and LDF could not be analyzed in all subjects, meaning that these results must be viewed as exploratory in nature. Finally, we used deionized water to dilute ACh and SNP in the iontophoresis experiments, which has since been shown to be able to induce some non-specific vasodilation compared to use of sodium chloride [251]. Since the methodology was standardized across the study, however, this is unlikely to have affected the results in any major way.

6 CONCLUSIONS

- Patients with type 1 diabetes have significantly higher levels of plasma MVs compared to matched healthy controls, but with no clear relationship between MV levels and the presence of clinical microangioapthy.

- Patients with type 1 diabetes have a pronounced increase in plasma EMVs, an indication of widespread endothelial activation and dysfunction, whereas plasma PMVs are only modestly elevated compared to healthy controls. HMGB1+ MVs were shown for the first time to be elevated in patients, with potential implications for future cardiovascular complications.

- Women with type 1 diabetes experience a disproportionate shift towards a more procoagulant MV phenotype compared to their male counterparts.

- We have for the first time identified plasma lactadherin as a potential biomarker of clinical microangioapthy in type 1 diabetes.

- We were able to demonstrate that single centrifugation of fresh samples, followed by re-centrifugation of thawed samples prior to flow cytometry analysis of MVs, is a valid and simple laboratory approach that might pave the way towards the use of MVs as clinically useful biomarkers in other proinflammatory and prothrombotic diseases.

- Patients with type 1 diabetes have diminished skin microvascular reactivity and the magnitude of these changes reflect the severity of their microvascular complications. Our findings support the use of skin microcirculation as a useful modell to study microangiopathy.

7 POINTS OF PERSPECTIVE

This thesis demonstrated that perturbations in skin microvascular reactivity reflect the degree of clinical microangiopathy in type 1 diabetes. In the future, if measurements of skin microcirculation can be further validated as an informative approach for studying microangiopathy, simplified bedside measurements of skin microcirculation could be implemented in the clinic as a non-invasive screening tool to help identify subclinical levels of vasculopathy. This would hopefully allow for intensified medical management to help prevent nephropathy, retinopathy, neuropathy as well as heart disease and stroke.

Importantly, our study looking at skin microvascular disturbances in type 1 diabetes identified a clear contribution of endothelial-independent pathways, which highlights the need to focus research not only on the endothelial cells themselves, but also on smooth muscle cells as well as the surrounding vascular tissue. Tissue remodeling and fibrosis of the extracellular matrix surrounding the microvasculature may both play important roles in microvascular function. Fibrosis has been identified as a common pathway for the progression of kidney disease regardless of origin [203]. Another key component of the microcirculation is the glycocalyx, the 20-100 nm layer of negatively charged glycosylated proteins covering the luminal surface of the vessel wall, which has been shown to play a crucial role in maintaining microvascular permeability as well as helping to prevent micro-thrombi [252]. The glycocalyx is damaged under inflammatory and hyperglycemic conditions, leading to increased vascular permeability and shedding of major glycocalyx components into the blood and urine [253]. Young patients with type 1 diabetes without manifest vascular complications have a thinner glycocalyx lining than healthy controls [254]. Loss of glycocalyx integrity in the kidneys leads to increased microvascular permeability to albumin [90] and glycocalyx measurements can differentiate between patients with or without microalbuminuria [255]. The use of handheld devices employing incident dark field video-microscopy for direct bedside assessment of the glycocalyx in the sublingual microvasculature is also under study, although results with regards to their clinical utility have been somewhat conflicting thus far [256, 257].

We identified lactadherin as a potentially novel biomarker of microvascular complications in type 1 diabetes, which must now be replicated and validated in larger studies. It would also be of interest to study plasma lactadherin levels in other chronic proinflammatory conditions with potentially accelerated cellular turnover in the circulation, such as type 2 diabetes, rheumatological conditions, and chronic kidney disease.

Despite not finding a clear link between MV levels and clinical microangiopathy in this thesis, the highly elevated levels of EMVs identified in patients are still of interest, as they demonstrate that type 1 diabetes per say is a proinflammatory condition associated with a high degree of endothelial activation and dysfunction. Inflammation, endothelial dysfunction and hemostatic disturbances have been identified as shared pathogenic mechanisms driving both microangiopathy and macrovascular disease [110], and in the future it would therefore be

worthwhile to prospectively study whether elevated MV levels in type 1 diabetes may have prognostic value for future cardiovascular disease.

Increased HMGB1+ MVs in patients with type 1 diabetes is significant given the role of HMGB1 as a potent proinflammatory alarmin contributing to endothelial dysfunction, which again may have implications for future macrovascular disease. Future studies will also have to assess the biological function of these HMGB1+ MVs, since HMGB1 can exist in different redox states that affect its biological activity [258]. HMGB1+ PMVs have recently been shown to induce endothelial dysfunction in systemic sclerosis through a NET-dependent mechanism [259]. Given that NETs have emerged as drivers of both microvascular as well as macrovascular complications in type 1 diabetes [260], it would also be valuable to evaluate the relationship between HMGB1+ MVs and NETosis in type 1 diabetes more closely.

In a small pilot study, we found a significant negative correlation between PS+ HMGB1+ PMVs and total HMGB1+ PMVs and ACh-dependent microvascular flux, as well as a trend towards a negative association to SNP-dependent microvascular flux. It would be interesting to study the correlation between HMGB1+ PMVs and skin microcirculation in a larger study.

A promising field of biomarker research is the study of exosomes and their miRNA content in relation to microvascular complications in type 1 diabetes, where smaller studies have already identified a few promising candidates [261, 262]. The use of urinary MVs for improved detection of diabetic nephropathy might also allow for non-invasive disease monitoring [263-265]. In an ideal world, extracellular vesicles (MVs and exosomes) from either plasma or urine could be used a sort of liquid biopsy [174] for risk-stratification to improve and personalize the treatment of patients. Research has shown that a subset of patients with type 1 diabetes never develop microvascular complications, regardless of clinical risk factors [49]. Future research will have to evaluate whether MVs could help identify such low-risk individuals, in the hope of being able to avoid excessive medical treatment with potential side effects.

Ongoing methodological and technical advances in the field of flow cytometry will in the future hopefully allow for even more accurate detection of MVs as well as smaller exosomes in plasma and urinary samples. However, it is of key importance that a balance is struck such that developments in the MV field are not hindered by methodological puritanism. Pre-analytical handling and laboratory protocols need to be simple enough to allow for plasma samples to be collected in a clinical setting, such that translational MV studies can focus on answering relevant research questions that may ultimately serve to directly benefit patients. Only then can MVs be implemented in a clinical setting and achieve their full potential as biomarkers.

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