Aus der Klinik für Neurologie mit Experimenteller Neurologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Circulating Microvesicles and Cardiovascular Risk after Ischemic Stroke

Zirkulierende Mikrovesikel und kardiovaskuläres Risiko nach ischämischem Schlaganfall

zur Erlangung des akademischen Grades
Medical Doctor/Doctor of Philosophy (MD/PhD)

vorgelegt der Medizinischen Fakultät
Charité – Universitätsmedizin Berlin

von

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List of Abbreviations

CD cluster of differentiation.

CI confidence interval.

EMV endothelial microvesicles.

EPCAS Endothelial Progenitor Cells in Acute ischemic Stroke.

HR hazard ratio.

IQR interquartile range.

LMV leukocyte-derived microvesicles.

MI myocardial infarction.

MMV monocytic microvesicles.

mRS modified Rankin Scale.

MV microvesicles.

NIHSS National Institutes of Health Stroke Scale.

NMDAR1-abs anti-N-Methyl-D-Aspartate receptor GluN1 antibodies.

NO nitric oxide.

OR odds ratio.

PMV platelet-derived microvesicles.

PROSCIS PROSpective Cohort with Incident Stroke.

PROSCIS-B PROSpective Cohort with Incident Stroke Berlin.

PROSCIS-M PROSpective Cohort with Incident Stroke Munich.

PS phosphatidylserine.

TF tissue factor.

TMAO trimethylamine N-oxide.

TOAST Trial of Org 10172 in Acute Stroke Treatment.

Abstract in English

Microvesicles are released by various cell types upon activation by outward-blebbing of the cell membrane and contain surface markers and contents of the parent cell. In an experiemental setting, endothelial microvesicles (EMV) have been shown to mediate endothelial dysfunction. Clinical studies have shown an association with cardiovascular risk factors and poor outcome in cardiovascular diseases such as myocardial infarction. In ischemic stroke patients, as well as other neurological diseases, microvesicle (MV) levels are elevated after the acute event compared to healthy controls. The potential prognostic role of circulating EMV and MV of other origins for long-term cardiovascular outcome after ischemic stroke has not yet been studied.

The PROSpective Cohort with Incident Stroke Berlin (PROSCIS-B) is an observational cohort study enrolling patients with first-ever ischemic stroke with a follow-up period of three years. The primary combined endpoint was predefined as recurrent stroke, myocardial infarction (MI), and all-cause mortality. Citrate-blood samples were taken on day 4 (IQR 3-5) after stroke. Levels of endothelial (Annexin V+ CD45- CD41- CD31+/CD144+/CD146+), leukocyte-derived (LMV) (Annexin V+ CD45+ CD41-), monocytic (MMV) (Annexin V+ CD14+ CD41-), and platelet-derived microvesicles (PMV) (Annexin V+ CD41+) were measured using flow cytometry. Kaplan-Meier survival analysis, the log-rank test and Cox proportional hazards models were used to calculate the impact of MV levels on the combined endpoint within three years after adjustment for potential confounding variables.

Between January 2010 and June 2013, 621 patients with mild to moderate stroke were recruited of whom 571 had MV measurements (median age 69 years, 39% female, median National Institutes of Health Stroke Scale (NIHSS) 2, interquartile range (IQR) 1-4). During the follow-up period, 95 endpoints (42 recurrent strokes, 5 MI and 48 deaths) occurred. Ischemic stroke patients with levels of EMV (hazard ratio (HR) 2.5, 95% confidence interval (CI) 1.2-4.9) or LMV (HR 3.1, 95% CI 1.4-6.8) in the top quartile suffered more often a combined endpoint than participants with lower levels. This relationship was weaker for PMV (HR 1.7, 95% CI 0.9-3.2) and for MMV (HR 1.1, 95% CI 0.6-1.8) it was not detectable.

In the present study, high levels of EMV and LMV were associated with an increased incidence of secondary cardiovascular events or death after mild to moderate ischemic stroke in the long term. These results enhance the significance of endothelial dysfunction and vascular inflammation for post-stroke outcome. EMV and LMV therefore might be potential cofactors for risk prediction after ischemic stroke.

Abstract auf Deutsch

Mirkovesikel werden von unterschiedlichen Zelltypen bei Aktivierung durch Ausstülpung der Zellmembran freigesetzt und enthalten Oberflächenmarker und teilweise Inhalte der Ursprungszelle. Endotheliale Mikrovesikel sind durch experimentelle Studien als Surrogatmarker für endotheliale Dysfunktion etabliert und in kardiovaskulären Erkrankungen wie dem Myokardinfarkt mit schlechtem Outcome assoziiert. In der Akutphase ischämischer Schlaganfälle sind Mikrovesikelkonzentrationen verglichen zu gesunden Kontrollen deutlich erhöht und zeigen einen Zusammenhang mit Schlaganfallschwere. Die Bedeutung zirkulierender Mikrovesikel verschiender Ursprungszellen im kardiovaskulären Langzeit-Outcome nach ischämischem Schlaganfall ist derzeit noch unklar.

In der Studie PROSpective Cohort with Incident Stroke Berlin (PROSCIS-B) wurden PatientInnen mit erstmaligem ischämischen Schlaganfall für drei Jahre beobachtet. Der vordefinierte primäre kombinierte Endpunkt beinhaltete Rezidivschlaganfälle, Myokardinfarkte und Gesamtmortalität. Die Konzentrationen von endothelialen (Annexin V+ CD45- CD41- CD31+/CD144+/CD146+), leukozytären (Annexin V+ CD45+ CD41-), monozytären (Annexin V+ CD14+ CD41-) und thrombozytären Mikrovesikeln (Annexin V+ CD41+) wurden mittels Durchflusszytometrie im Median am 4. Tag (IQR 3-4) nach Schlaganfall im Citratplasma bestimmt. Zur Berechnung der Effektstärke von Mikrovesikeln auf den kombinierten Endpunkt innerhalb drei Jahre wurden die Kaplan-Meier Überlebensanalyse, der Log-Rank Test und die adjustierte Cox Regressionsanalyse angewendet.

Zwischen Januar 2010 und Juni 2013 wurden 621 Patienten mit milden bis moderaten Schlaganfällen rekrutiert, von denen 571 Mikrovesikelmessungen erhielten (medianes Alter 69 Jahre, 39% weiblich, medianer NIHSS 2, IQR 1-4). Im Beobachtungszeitraum traten 95 Endpunkte (42 sekundäre Schlaganfälle, 5 Myokardinfarkte, 48 Todesfälle) auf. Schlaganfallpatienten mit Konzentrationen in den höchsten Quartilen von endothelialen (HR 2.5, 95% CI 1.2-4.9) oder leukozytären Mirkovesikeln (HR 3.1, 95% CI 1.4-6.8) erlitten häufiger einen kombinierten Endpunkt als Patienten mit niedrigeren Konzentrationen. Dieser Zusammenhang war für thrombozytäre Mirkovesikel weniger deutlich (HR 1.7, 95% CI 0.9-3.2) und für monozytäre nicht erkennbar (HR 1.1, 95% CI 0.6-1.8).

In dieser Studie waren hohe Konzentrationen endothelialer und leukozytärer Mikrovesikel mit einer langfristig erhöhten Inzidenz sekundärer kardiovaskulärer Ereignisse oder Tod nach mildem bis moderatem Schlaganfall assoziiert. Diese Ergebnisse verdeutlichen die Signifikanz endothelialer Dysfunktion und vaskulärer Inflammation für das Outcome nach Schlaganfall. In der Risikoprädiktion nach ischämischem Schlaganfall könnten endotheliale und leukozytäre Mikrovesikel daher potenziell eine wichtige Rolle spielen.

1 Introduction

1.1 Stroke as a global health burden

Stroke is a cerebrovascular disease with acute onset, mostly causing permanent neurological deficits, and also the second most common cause of death and disability worldwide.[1] In 2017, 67% of strokes were ischemic, caused by a temporary or irreversible reduced perfusion due to cerebral artery occlusion.[1] After a first stroke, cardiovascular risk for recurrent events remains especially high in the first year, but also significantly increased in the long-term (2^{nd} through 5^{th} year), indicating that secondary prevention can play an important role for the long-term clinical outcome.[2] In addition to commonly known clinical cardiovascular risk factors (atrial fibrillation, hypertension, diabetes), biomarkers have proven as valuable tools for risk prediction and for further understanding of the underlying pathomechanism in disease progression.

1.2 The biology of microvesicles

Microvesicles (MV), formerly known as microparticles, are a subtype of extracellular vesicles in size of $100nm$ to $1\mu m$ formed by outward-blebbing of the cell membrane. Potentially, they can be generated by any cell type and underlie a dynamic balance between formation and clearance. Besides physiological processes such as growth, differentiation and apoptosis, other mechanisms that induce MV formation are related to non-physiological cell stress, e.g. caused by hypoxia, cytokines, shear stress and many more.[3] The stimulated cell undergoes reorganization of the membrane phospholipids, leading to exposure of phosphatidylserine (PS) on the outer leaflet following the dysregulation of scramblase, floppase and flippase activities.[4, 5] Thus, they carry the same surface markers and molecular components of the parent cells and show PS on the outer leaflet of the vesicle membrane. What was first described in 1967 as "platelet-dust"[6], procoagulant side-products of an activated cell, proved to be way more than that. When released into the bloodstream, we now know that circulating MV can act as signaling structures between cells, transporting RNA, metabolites and proteins, contributing to systemic effects such as immune function and inflammation. Notably, MV are a very heterogeneous population and their components can vary by their parent cell or by stimulus type.[3] MV can be found not only in blood but also in various biofluids such as cerebrospinal fluid, saliva, urine and breast milk. Today, they are seen as surrogate for mediating pathomechanisms, as biomarkers, and both as therapeutic agent and target.

MV are distinguished from other extracellular vesicles (exosomes, apoptotic bodies) by size and biogenesis, see fig. 1 and tab. 1 for an overview about vesicle types and their characteristics.

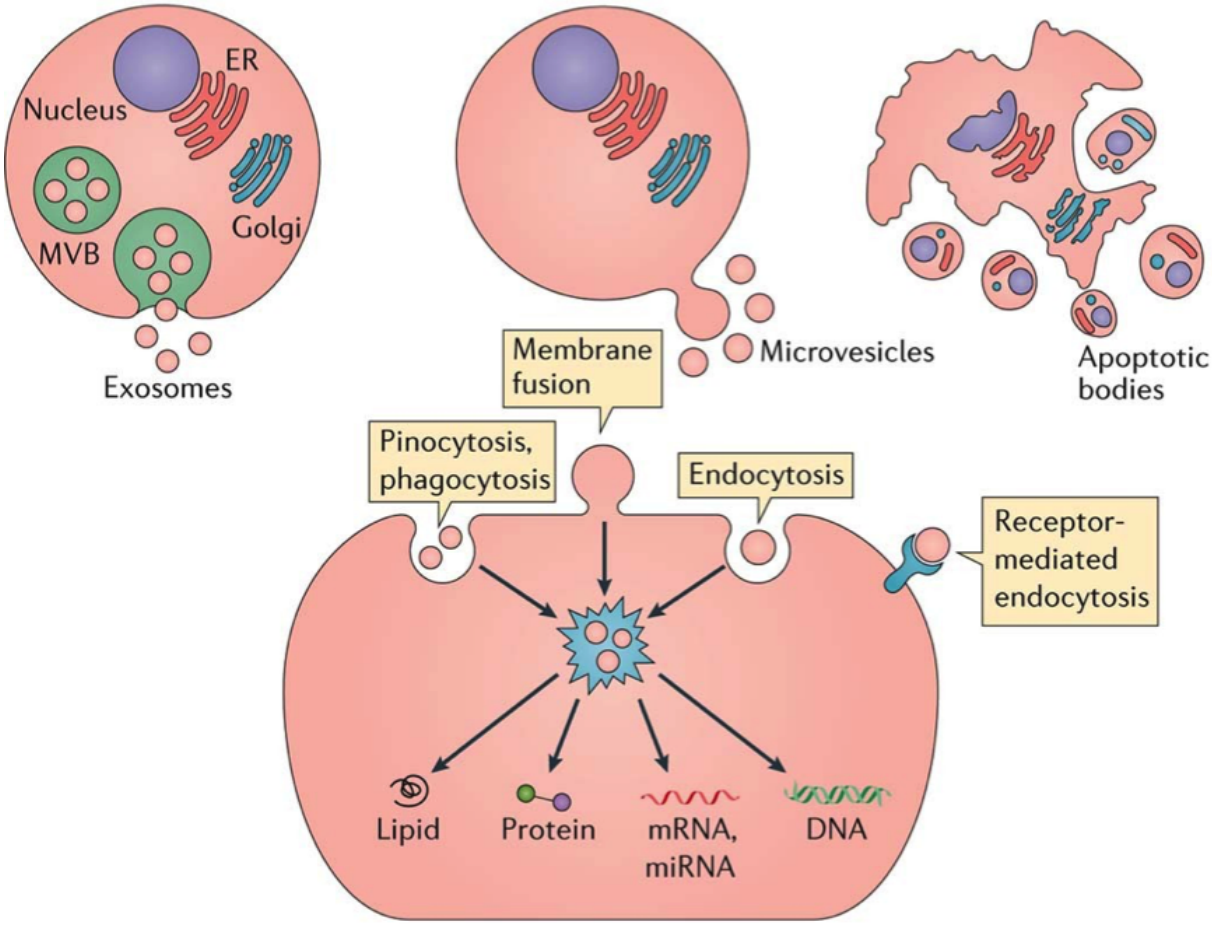

Figure 1: **Classes of extracellular vesicles and their release and uptake mechanisms.** According to their biogenesis pathways, extracellular vesicles are classified as exosomes, microvesicles and apoptotic bodies. The uptake into the recipient cell is mediated by endocytosis, phagocytosis, pinocytosis, or membrane fusion. Extracellular vesicles carry ligands on their surface that can bind to a receptor on the target cell and further activate an intracellular signal cascade.[7] ER = endoplasmic reticulum, miRNA = microRNA, MVB = multivesicular body, rRNA = ribosomal RNA

Vesicle types	Origin	Size (nm)	Markers	Content
Exosomes	Endolysosomal pathway; intraluminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane	40–120	Tetraspanins (such as TSPAN29 and TSPAN30), ESCRT components, PDCD6IP, TSG101, flotillin, MFGE8	mRNA, microRNA (miRNA) and other non-coding RNAs; cytoplasmic and membrane proteins including receptors and major histocompatibility complex (MHC) molecules Microvesicles
Microvesicles	Cell surface; outward budding of cell membrane	50–1,000	Integrins, selectins, CD40 ligand	mRNA, miRNA, non-coding RNAs, cytoplasmic proteins and membrane proteins, including receptors
Apoptotic Bodies	Cell surface; outward blebbing of apoptotic cell membrane	500–2,000	Extensive amounts of phosphatidyl- serine	Nuclear fractions, cell organelles

Table 1: **Characteristics of extracellular vesicle subtypes.** Extracellular vesicles are classified by their release mechanisms, as depicted in fig. 1 and described in the table above. The characteristic markers presented here, however, are not exclusively specific, so that they only summarize which ones are more common on one vesicle type than on others.[8]

1.3 Microvesicles as biomarkers in cardiovascular diseases

In cardiovascular diseases, MVs have been found to pathophysiologically contribute to thrombus formation, hypercoagulation, endothelial dysfunction and vascular inflammation. In atherosclerosis, there is evidence for platelet-derived microvesicles (PMV), endothelial microvesicles (EMV) and monocytic microvesicles (MMV) to accelerate disease progression via various pathways.[9] EMV can inhibit nitric oxide (NO) synthase leading to a decline in NO production and consequently vascular dysfunction and impaired vascular relaxation.[10, 11] Leukocyte-derived microvesicles (LMV) and EMV isolated from atherosclerotic plaques were increased in numbers and thrombogenic activity.[12] In vivo, associations of circulating MV and cardiometabolic risk factors have been observed, for example in a large cohort such as the Framingham Heart study.[13] In myocardial infarction (MI), MV were correlated to lesion size and severity and in stable coronary artery disease, they proved as independent predictors of cardiovascular long-term outcome.[14, 15, 16]

Fig. 2 summarizes the mechanisms involved in MV formation and their effects in the target tissue relevant for cardiovascular diseases.

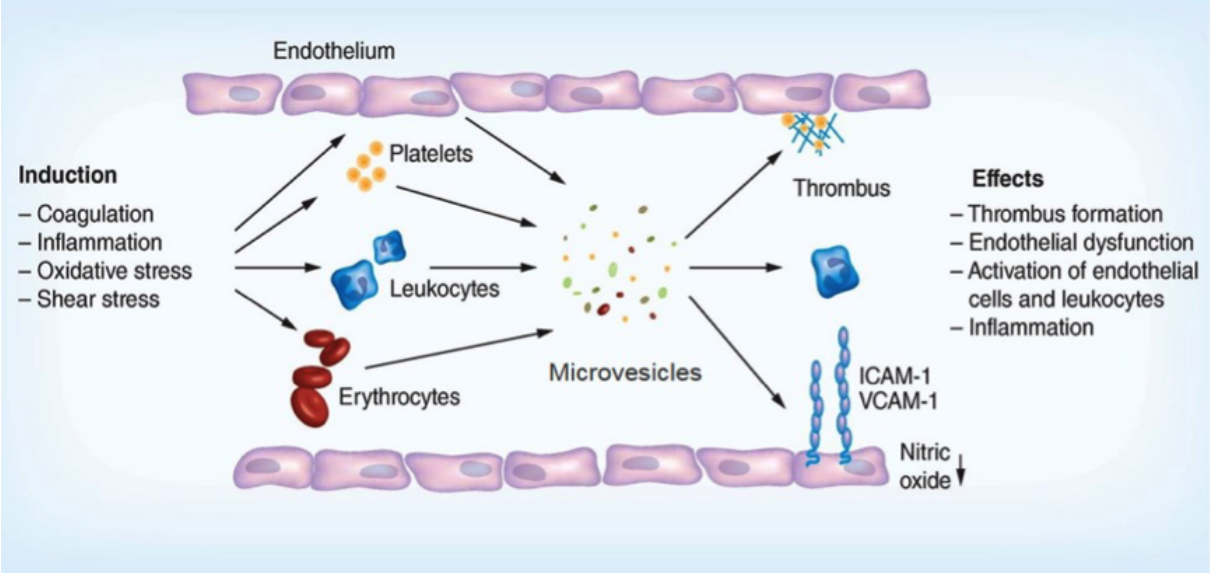

Figure 2: **Induction of MV formation and their effects in the cardiovascular system.** In the cardiovascular system, MV formation can be triggered by coagulation, inflammation, oxidative stress or shear stress, among others. When released into the bloodstream, they bind to the target cell causing thrombus formation, endothelial dysfunction and inflammation by upregulation of adhesion molecules and reducing NO production.[17]

Corresponding to the findings in the field of cardiology, there has been increasing effort to gain knowledge about the role of MV in neurovascular diseases. Yun et al. have summarized in their review that in patients with Alzheimer's disease, multiple sclerosis and traumatic brain injury, MV levels are higher compared to healthy controls and negatively linked to disease severity and clinical outcome.[18] With regard to the shared risk factors of ischemic stroke and MI, researchers have extrapolated the knowledge on MV in stroke patients. In the acute phase after ischemic stroke, EMV, LMV, PMV and MMV are elevated and correlate to disease etiology, lesion size and clinical severity.[19]

1.4 Aim

In our study, we aimed to investigate the impact of MV levels on long-term cardiovascular outcome after stroke. MV could play a role as surrogate for vascular injury during ischemic stroke and mediate adverse effects on vascular regeneration and endothelial function.

1.5 Context of the study group

The PROSpective Cohort with Incident Stroke (PROSCIS) is a longitudinal, prospective, hospital-based, observational cohort study conducted independently in two centers: Berlin (PROSCIS-B) and Munich (PROSCIS-M). The study protocol has been published previously.[20] PROSCIS aims to find a model predicting the long-term secondary cardiovascular risk after incident stroke. Besides classical cardiovascular risk factors, we also assess both the causal role and the predictive value of novel biomarkers for outcome after stroke. While in this work, the data of PROSCIS-B was used, PROSCIS-M is planned as a validation cohort. At the time this text was written, the PROSCIS-M cohort was still recruiting, so that a validation of our findings is still pending.

In previous publications, we investigated the influence of anti-N-Methyl-D-Aspartate receptor GluN1 antibodies (NMDAR1-abs)-seropositivity, Gut microbiota-dependent trimethylamine N-oxide (TMAO) and coagulation factor XII, XI, and VIII activity levels on cardiovascular long-term outcome after stroke.[21, 22, 23] Further biomarkers under investigation include lipoprotein A, high-sensitivity cardiac troponin T and high-sensitivity CRP. By identification of crucial biomarkers in combination with classical cardiovascular risk factors, we hope to be able to generate an advanced prediction model for cardiovascular long-term outcome after stroke.

Secondary outcomes that are under investigation are functional outcome, cognitive function and depression. Below, I will briefly summarize the content of two published works that I have co-authored.

1.5.1 Serum Anti-NMDA (N-Methyl-D-Aspartate)-Receptor Antibodies and Long-Term Clinical Outcome After Stroke (PROSCIS-B)

Pia S. Sperber, Bob Siegerink, Shufan Huo, Jessica L. Rohmann, Sophie K. Piper, Harald Prüss, Peter U. Heuschmann, Matthias Endres, Thomas G. Liman

doi: 10.1161/strokeaha.119.026100 [21]

In this publication, we investigated the impact of NMDAR1-abs on long-term clinical outcome after stroke. In the field of neurology, NMDAR1-abs are mostly known for their pathogenic role in autoimmune encephalitis, but they can also be found in serum of approximately 10% of healthy population.[24] The clinical significance of NMDAR1-abs seroprevalence in healthy individuals remains unclear. Previous studies suggested a protective effect in stroke because antibody-presence was associated with smaller lesion size in magnetic resonance imaging.[25] It is suspected that excitotoxicity mediated by NMDARs (N-Methyl-D-Aspartate receptors) plays a major role in tissue loss.

We measured NMDAR1-abs IgG, IgA and IgM in patient serum from PROSCIS-B during the first week after stroke. Functional outcome was quantified by modified Rankin Scale (mRS) at one year and odds ratios (ORs) were calculated by partial proportional odds models. In a next step, seropositive patients were divided into groups with high ($1:320$; $1:1000$) and low ($1:10; 1:32$; and $1:100$) titers and compared. Cardiovascular outcome was evaluated by a combined endpoint (recurrent stroke, MI, death) during three years of follow-up. We used an adjusted Cox proportional Hazards regression model to estimate hazard ratios in a survival analysis.

In total, 583 patients with antibody-measurements were included. NMDAR1-abs were measured in 76 (13%) of these patients. 96 combined endpoints occurred during the follow-up period. In contrast to the previous results, we found that NMDAR1-ab seropositivity was not associated with improved functional outcome (OR 1.27, 95%CI 0.77–2.09) or cardiovascular risk. In fact, we discovered that NMDAR1-ab seropositivity was associated with poor cardiovascular outcome (HR 1.83, 95%CI 1.10–3.05) and high titers ($\geq 1:320$) with worse functional outcome after one year (OR 3.47, 95%CI 1.54–7.80). A possible explanation for this finding is that after disruption of the blood-brain barrier, NMDAR1-abs are able to access brain tissue and downregulate NMDAR. In the long term after stroke, this could impair functional recovery and increase vascular risk.

1.5.2 Coagulation factor XII, XI, and VIII activity levels and secondary events after first ischemic stroke

Jessica L. Rohmann, Shufan Huo, Pia S. Sperber, Sophie K. Piper, Frits R. Rosendaal, Peter U. Heuschmann, Matthias Endres, Thomas G. Liman, Bob Siegerink

doi:10.1111/jth.15092 [23]

In this project, we studied the association between activity levels of coagulation factors XII, XI, and VIII and cardiovascular outcome after stroke. In previous studies, FXI activity has been related to worse outcome as in stroke severity, mRS and Barthel Index at discharge.[26] Elevated FVIII activity in ischemic stroke patients yielded a higher occurrence of recurrent thrombotic events. While FXII seems to be a risk factor for first-ever vascular events in animal studies, its role in ischemic events in humans remains unclear.[27]

In this study, we measured coagulation factor activity levels in citrate blood samples of PROSCIS-B patients using a one-stage clotting assay. Again, the combined cardiovascular endpoint (secondary stroke, MI and all-cause death) was used in a Cox proportional Hazards regression analysis to assess cardiovascular outcome.

In total, 576 patients had at least one of three coagulation factor measurements and for 553 patients, all three factor activity level measurements were performed. 94 combined endpoints occurred during the follow-up period. We found that high levels of FVIII (HR 2.05, 95%CI 1.28-3.29) or FXI activity (HR 1.80, 95%CI 1.09-2.98) were associated with an increased cardiovascular risk. High FXII activity levels did not show an association with a changed hazard for the combined endpoint (HR 0.86, 95%CI 0.49-1.51). Given previous literature, in which reduced FXI activity in Mendelian randomization yielded a lower risk of venous thrombosis and ischemic stroke, our findings suggest a possible benefit of antithrombotic therapy targeting FXI for long-term cardiovascular outcome of stroke patients.[28]

2 Supplementary Methods

For a detailed description of the methodology of the main work please see "Methods" section of the publication Huo et al.[29] In the following, I will elaborate more on methodology not mentioned in the paper.

2.1 Surface markers for flow cytometry characterization

For detailed description of sample preparation, antibody staining procedures and flow cytometry measurements please see Huo et al.[29] Additionally, a detailed overview of the surface markers used in flow cytometry and their typical expression on cells is provided below (tab. 2). This table was given in the online supplementary material of our publication.[29]

Antigen	Target Structure Synonyms	Expressed on	Fluorochrome	Manufacturer
AV	Phosphatidylserine	All cells during early apoptosis	PerCP/Cy5.5	Biolegend
CD41	integrin α2b/GPIIb	Platelets and megakaryocytes	APC/Cy7	Biolegend
CD45	leukocyte common antigen (LCA)/T200	Hematopoietic cells, except circulating erythrocytes and platelets	BV711	Biolegend
CD14	LPS receptor	Myeloid cells (monocytes, macrophages, granulocytes)	BV421	Biolegend
CD31	platelet endothelial cell adhesion molecule (PECAM-1), EndoCAM	Endothelial cells, platelets, granulocytes, monocytes/macrophages, dendritic cells, and T and B cell subsets	BV605	Biolegend
CD144	Vascular endothelial-cadherin	Endothelial cells	PE	Biolegend
CD146	S-Endo 1 antigen, MUC18, MCAM, Mel-CAM, A32 antigen	vascular endothelial cells, a subset of NK1.1+ cells, and neutrophils	AF488	Biolegend

Table 2: Detailed characterization of flow cytometry surface markers and their expression on cells.

From the above table it is evident, that despite their well-established use in flow cytometry, these surface markers are not cell-specific but rather expressed on various cell types. To improve the distinction between MV populations of different origins, sequential gating combining inclusion and exclusion markers was applied as explained in the methods section of our publication.[29]

2.2 Developing the laboratory protocol

I used citrate-buffered plasma samples from healthy controls to establish a gating protocol using single staining and fluorescent-minus-one controls. (For a detailed example of our gating strategy see Fig. 1 in Huo et al.[29]) I tested the surface markers and gating procedure by positive controls with MV from the supernatant of cultured human aortic endothelial cells (HAEC), induced pluripotent stem cell-derived endothelial cells (iPSC-EC) and human peripheral blood mononuclear cells (PBMC). I also used our staining panel on whole blood to test the affinity of the antibodies on cells (leukocytes, monocytes, platelets). I used fluorescent beads (Megamix-Plus SSC; BioCytex, Marseille, France) to determine the upper size cutoff of 1000nm. MV were defined as all particles binding AV <1000nm.

The lower detection limit of the flow cytometry device Attune NxT acoustic focusing cytometer (Thermo Fisher Scientific, Waltham, MA) was tested using beads in sizes 20, 100, 200, 500, 1000, and 2000 nm (Flow Cytometry Sub-micron Particle Size Reference Kit; Thermo Fisher Scientific). As seen in fig. 3, 20nm particles were not detectable, whereas all other bead sizes can be clearly distinguished. 2000nm beads were excluded in the predefined gate of particles smaller than 1000nm. Therefore, lower detection limit is about 100nm well applicable for MV measurements.

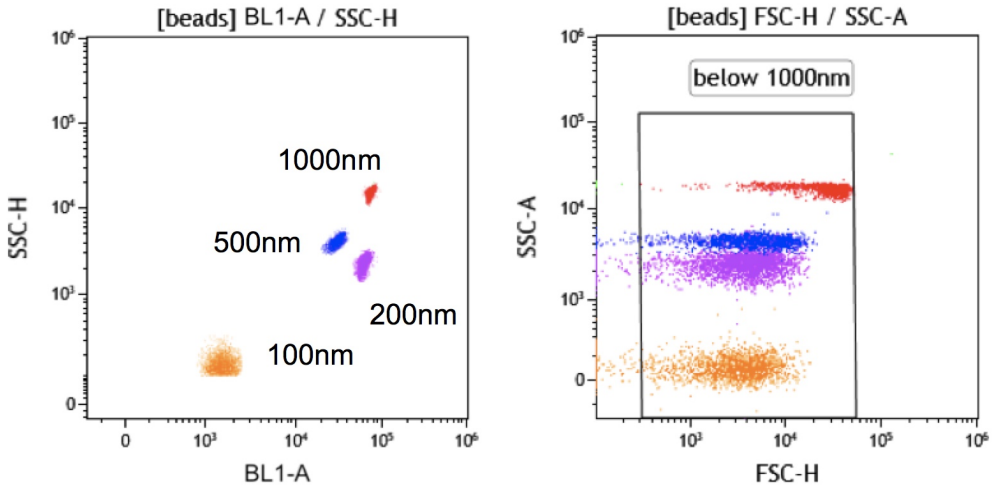

Figure 3: **Size calibration with Sub-micron Particle Size Reference Kit.** Beads are measured in flow cytometry and displayed according to their fluorescence (BL1-A) and their size (FSC). 20nm beads cannot be displayed, rendering a lower detection limit of about 100nm.
A = area, BL1 = blue channel 1, FSC = foward scatter, H = height, SSC = side scatter

Also, I tested the precision of the flow cytometry protocol by dividing plasma from one person after centrifugation into three samples and repeating the staining and measurement procedure on each sample separately. I then calculated the difference between each measurement of total MV count and MV subtypes yielding an overall coefficient of variation (CV) below 10% suggesting good comparability

between measurements.

To confirm that particles measured in flow cytometry are indeed lipid-membrane vesicles, I used the detergent Triton X-100 (final concentration 0.1%) on three samples and performed flow cytometry with and without Triton X-100. After this treatment, the previously visible AV+ population disappeared. Below, images of one sample before and after treatment with Triton X-100 are displayed (Fig. 4 and fig. 5).

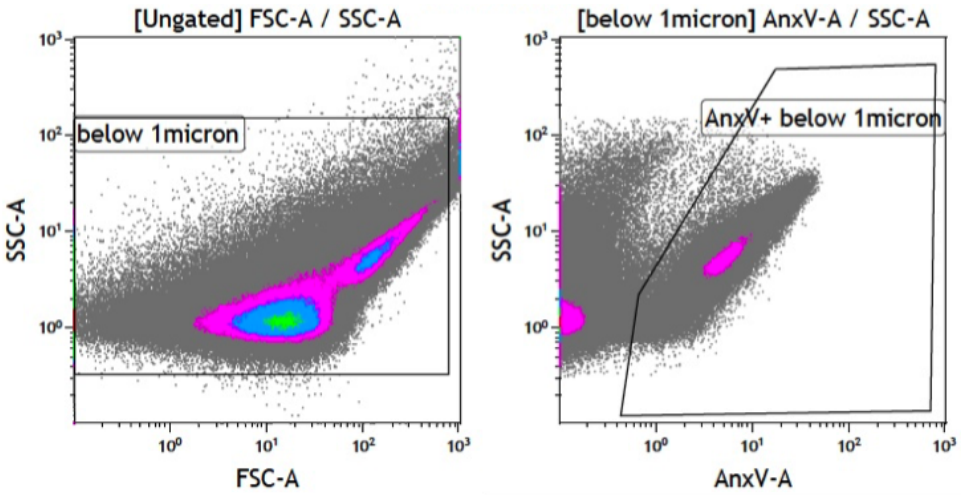

Figure 4: **Sample without Triton X-100.** A population positive for AnxV smaller than 1 μm is clearly visible. AnxV = Annexin V

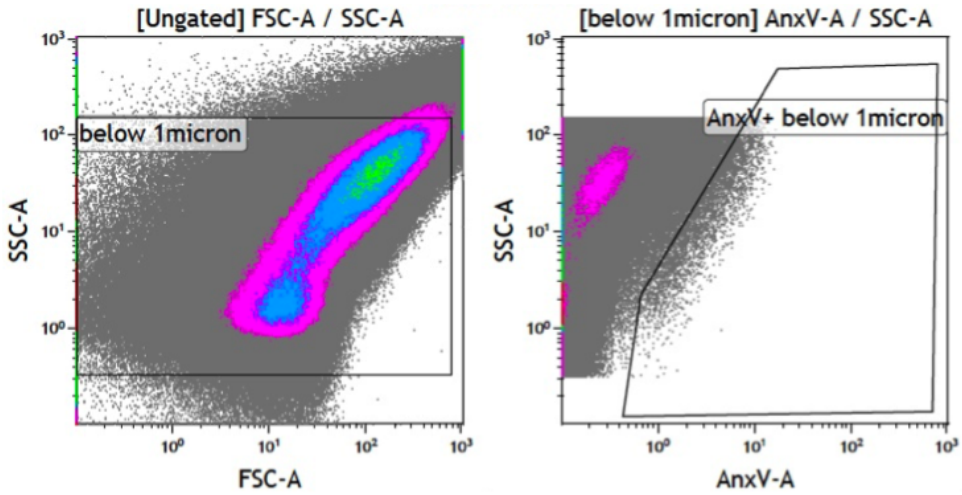

Figure 5: **Sample with Triton X-100.** After treatment with Triton X-100, the population has disappeared. Instead, more AnxV- particles are visible equivalent to debris.

2.3 Pilot study

To develop and verify the laboratory analysis protocol, I conducted a pilot study with patient samples from the study “Endothelial Progenitor Cells in Acute ischemic Stroke (EPCAS)", NCT01289795. In this study, patients were enrolled within 24 hours after first-ever ischemic stroke and subjected to subsequent blood draws on three time points after event onset (t1: day 1-2, t2: day 3-5, t3: day 6-7 or at discharge). Citrate-buffered blood was prepared in the same way as in the main protocol (see Huo et al. for details on experimental methodology)[29] and MV measurements were performed likewise.

2.4 Exploratory analyses in Pilot

To evaluate the differences between the three surface markers for EMV, I plotted the particles positive for each one of the markers against each other to see how well they correlate. Based on a good correlation between all 3, I decided to define EMV as MV positive for any of CD31, CD144 and CD146.

With the resulting levels of MVs I conducted a small exploratory analysis displaying the change in MV levels over time in the course of one week after stroke both in a boxplot and a spaghetti plot to demonstrate the change of MV over time overall and in each individual patient. I used a Shapiro-Wilk Test to test for normal distribution and a one sample Wilcoxon signed-rank Test for the Null Hypothesis that median $\Delta t3 - t1 = 0$.

3 Supplementary Results

For main results, please see our publication Huo et al.[29] Below, I will elaborate on results not included in the paper.

3.1 Pilot Study

In our pilot study, 11 patients with ischemic stroke have complete MV measurements at all three time-points. The median difference in EMV between $t1$ and $t3$ was $-0.26\mu l^{-1}$ (IQR $-1.43, 0.03$, p-value $= 0.08$). Detailed course of EMV levels are shown in Fig.6+7.

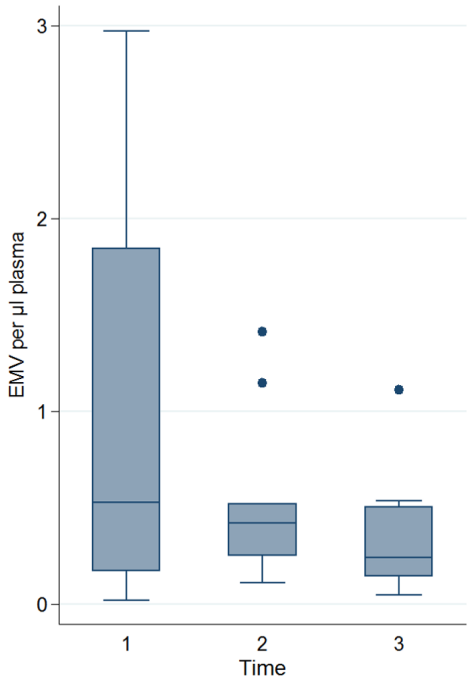

Figure 6: Course of endothelial microvesicle levels in the first week after ischemic stroke.

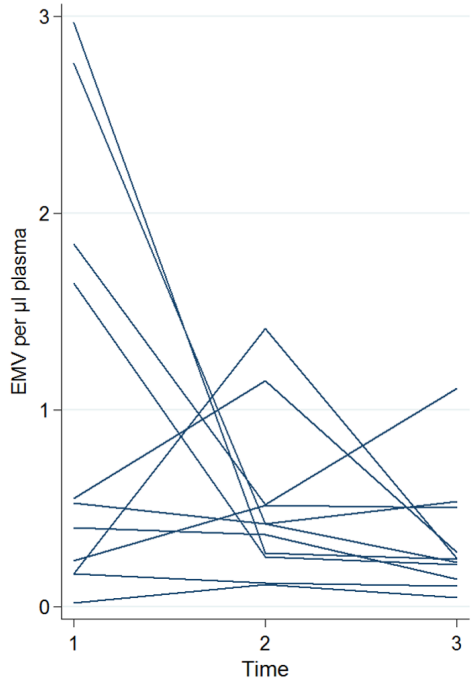

Figure 7: Changes in EMV levels in each individual in a spaghetti plot.

From these visualizations, it is evident that the concentration of EMV in citrate blood changes rapidly in the course of one week after the ischemic stroke. These exploratory findings suggest that EMV levels are most elevated in the early phase ($t1$ = day 1-2) after stroke and then decrease. Given that there is no literature describing the course of EMV levels in the acute phase after stroke, one can derive that the release and clearance of EMV during this period are highly dynamic processes. Similar changes were seen for LMV, PMV and MMV (results not displayed here). Although in our main study, the day of blood draw was not standardized (within 7 days after the index event), we tried to account for this phenomenon by adjusting for the number of days between stroke and blood draw.

3.2 Sample Size

Recruitment for PROSCIS-B was done between January 2010 and June 2013. Of 781 patients, who were screened, 690 gave written informed consent. 21 patients withdrew their consent, leaving 669 patients. 627 patients had suffered an ischemic stroke, while 42 had either a hemorrhagic stroke or sinus venous thrombosis. Only 6 patients had experienced a severe stroke with an NIHSS > 15 and were consequently not included in the final analysis due to sampling bias considerations. We managed to obtain data on MV measurements from 571 patients with our laboratory protocol, which is the final number of subjects in this analysis.

A total of 262 patients (46%) completed the 3 year follow-up period without suffering an endpoint or dropping out of the study. 216 patients (38%) were lost to follow-up but the majority of those contributed at least 1-2 years of person-time. We registered 95 combined cardiovascular endpoints consisting of 42 ischemic or hemorrhagic secondary stroke recurrences, 5 MI and 48 all-cause deaths.

Below, an extended flow chart of patient inclusion processes and number of endpoints/lost to follow-up at each year is displayed.

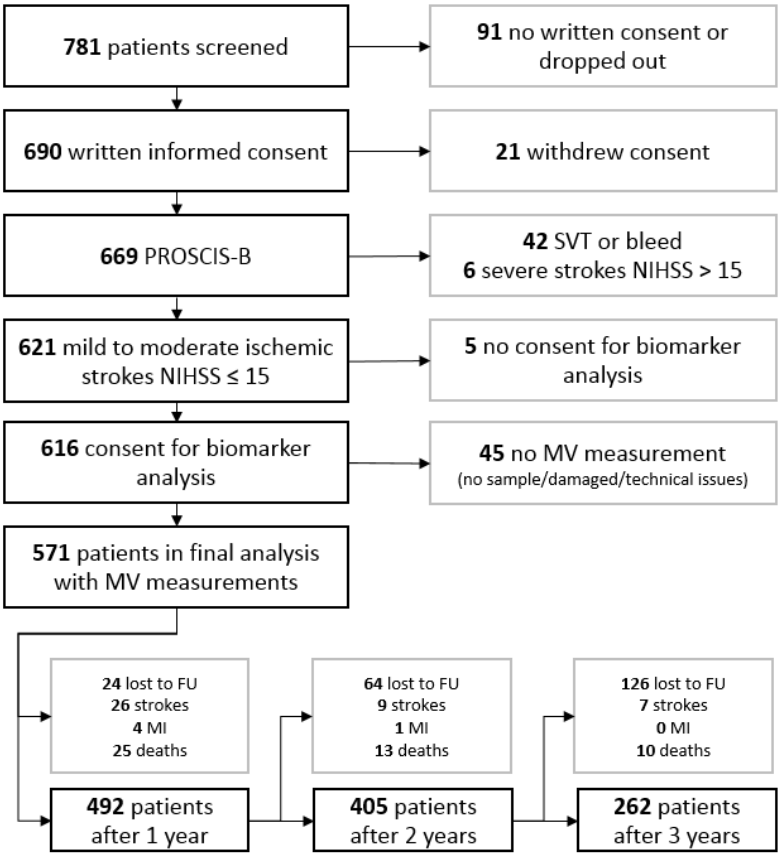

Figure 8: Patient inclusion/exclusion flowchart with patient numbers at each year of follow-up.

3.3 Adjusted Cox Regression

Supplementary to tables 3 and 4 from Huo et al.[29], I provide the results from the Cox Regression for all stages of adjustment. Due to limited space, in the publication only the results from model 3 were displayed.

EMV	HR0	95%CI	HR1	95%CI	HR2	95%CI	HR3	95%CI
Q1	1		1		1		1	
Q2	1.8	1.0-3.5	1.7	0.9-3.2	1.8	0.9-3.5	1.9	0.9-4.0
Q3	1.7	0.9-3.3	1.5	0.8-3.0	1.6	0.8-3.2	1.7	0.8-3.5
Q4	2.3	1.2-4.4	2.1	1.1-3.9	2.3	1.1-4.3	2.5	1.2-4.9

Table 3: Quartile Analysis for EMV

LMV	HR0	95%CI	HR1	95%CI	HR2	95%CI	HR3	95%CI
Q1	1		1		1		1	
Q2	3.4	1.7-6.8	3.3	1.6-6.7	3.3	1.6-7.0	3.8	1.7-8.2
Q3	1.9	0.9-4.0	1.6	0.7-3.3	1.5	0.7-3.4	1.7	0.7-3.9
Q4	3.3	1.6-6.6	2.5	1.2-5.1	2.8	1.3-6.0	3.1	1.4-6.8

Table 4: Quartile Analysis for LMV

PMV	HR0	95%CI	HR1	95%CI	HR2	95%CI	HR3	95%CI
Q1	1		1		1		1	
Q2	1.5	0.8-2.7	1.4	0.7-2.6	1.2	0.6-2.4	1.4	0.7-2.8
Q3	1.5	0.8-2.8	1.3	0.7-2.4	1.2	0.6-2.4	1.2	0.6-2.4
Q4	1.7	0.9-3.1	1.5	0.8-2.8	1.6	0.8-2.9	1.7	0.9-3.2

Table 5: Quartile Analysis for PMV

MMV	HR0	95%CI	HR1	95%CI	HR2	95%CI	HR3	95%CI
Q1	1		1		1		1	
Q2	0.7	0.3-1.3	0.7	0.4-1.4	0.8	0.4-1.5	0.8	0.4-1.6
Q3	0.9	0.5-1.6	0.9	0.5-1.6	1.0	0.5-1.8	1.0	0.5-1.8
Q4	1.2	0.7-1.9	1.0	0.6-1.7	1.1	0.6-1.8	1.1	0.6-1.8

Table 6: Quartile Analysis for MMV

HR0: crude model, HR1: adjusted for age, sex, NIHSS, HR2: additionally adjusted for Trial of Org 10172 in Acute Stroke Treatment (TOAST) and cardiovascular risk factors (smoking, alcohol consumption, hypertension, peripheral artery disease, atrial fibrillation, diabetes mellitus, MI), HR3: additionally adjusted for thrombolysis and number of days between stroke and blood draw.

Tables 3-6 show HR for quartile groups of MV subtypes with stepwise adjustment to confounders in all three models. Due to limited space, only model 3 was included in our publication, whereas HR0 - HR2 are solely displayed in the online supplement.

Through all levels of adjustment, the HR are robust.

4 Discussion

In this study, we could show that levels of circulating EMV (defined as AV+ CD31+/CD144+/CD146+ CD41- CD45-) and LMV (defined as AV+ CD45+ CD41-) are correlated to worse cardiovascular outcome 3 years after ischemic stroke. The relationship was not clearly detectable for PMV (defined as AV+ CD41+) and absent for MMV (defined as AV+ CD14+ CD41-). Concluding from the pilot analysis, I demonstrated that levels of circulating MV change rapidly during the acute phase (1 week) after stroke.

4.1 Microvesicles as biomarkers for long-term cardiovascular outcome after stroke

So far, most knowledge about the possible role of microvesicles in cardiovascular diseases originates from the field of cardiology; a short summary is provided in the introduction.

However, researchers have growing interest in expanding these findings in neurological diseases. A systematic review by Wang et al. summarizes what is known about the behavior of MV in stroke in a pooled meta-analysis: thirteen case-control studies have investigated levels of circulating MV in a total of 988 ischemic stroke patients compared to 985 healthy controls.[30] The pooled concentrations of EMV, LMV, PMV and MMV were significantly increased in stroke patients. Other investigators report correlations between EMV levels, NIHSS and infarct volume, short-term outcome[19, 31] and stroke etiology.[32]

The role of EMV in long-term clinical outcome after stroke has been studied by Lee et al. in a cohort of 298 ischemic stroke patients who were followed for 36 months.[33] As opposed to our study, they measured EMV levels not in the acute phase but only ≥ 3 months after the index event. They found that higher levels of CD62E+ EMV were associated with higher rates of cardiovascular events whereas high CD31+AV+ levels were not. Our study expands these findings in a larger cohort and in MV of other origins. Additionally, bearing in mind that CD31+ is expressed both on EMV and PMV, we used a more specific phenotyping for MV of endothelial origin (AV+ CD31+/CD144+/CD146+ CD41- CD45-) that allows us to exclude MV of platelet-derived origin (CD41+).

Another recent study by Lundström et al. divided MV into several subpopulations and investigated their correlation to long-term cardiovascular outcome in a cohort of 211 stroke patients.[34] Specifically, patients with elevated PS-p-selectin+ PMV (CD41+ lactadherin-FITC- CD62P+) had a HR of 1.86 (95%CI 1.04–3.31) for the primary combined outcome of recurrent ischemic stroke or MI, whereas PS+p-selectin+ PMV (CD41+ lactadherin-FITC+ CD62P+) and PS+TF+ MV (lactadherin-FITC+ CD142+, might be platelet-derived or monocytic origin) showed no association with increased cardiovascular risk after full adjustment to confounding variables. These results are in line with ours regarding that we also did not find a significant correlation between PMV and cardiovascular outcome. However, we have not investigated PS- MV populations as to current knowledge, their significance in stroke remains unclear. The authors of Lundström et al. do not offer an explanation how to differentiate PS- MV from debris as PS is considered as marker for MV formation.

However, a direct comparison between studies has to be regarded with caution. A major challenge

in the studies of MV has been the lack of methodological standardization for MV measurements. This includes time of blood draw, sample treatment (centrifugation protocols, storage, transport) choice of surface markers for vesicle phenotyping and gating strategies.[35, 36, 37] Therefore, the European Society of Cardiology (ESC) and the International Society for Extracellular Vesicles (ISEV) have published recent consensus documents on which we based our methodology.[38, 39, 40]

Notably, as Wang et al. pointed out, in such a clinical study setting it is impossible to discriminate if MV elevation is cause or outcome of the cardiovascular event.[30] This is why clinical studies investigating in vivo MV levels including ours can only report associations between MV levels and clinical observations, but assumptions about their role in pathophysiology of the disease remain speculative.

The first known characteristic of MV was their procoagulant activity. Their surface is negatively charged due to PS expression and attracts positively charged coagulation factors VII, IX, X and prothrombin. This effect is further increased by presence of tissue factor (TF) expression, binding FVIIa and promoting activation of FX and FXI.[41] The expression of TF is increased in atherosclerotic plaques and relates to their progression.[41] Leroyer et al. isolated EMV and LMV from atherosclerotic plaques and found that the numbers of MV were at least 200-fold increased compared to numbers from plasma.[12] Besides, more than 50% of MV expressed TF and were highly thrombogenic.

EMV contribute to endothelial dysfunction by reducing NO concentration, inducing vascular inflammation, promoting coagulation and regulating vascular tone, angiogenesis and apoptosis.[42, 43] LMV participation in inflammation is mediated by interaction between immune cells, transporting aminophospholipids and promoting the release of cytokines.[44] Although the pathophysiological effects cannot be clearly assigned to a certain MV subtype, EMV are widely seen as key biomarkers for endothelial activation and dysfunction, LMV for vascular inflammation and PMV for thrombotic state.[44, 45]

Thus, endothelial dysfunction and vascular inflammation represented by high levels of EMV and LMV in the acute phase after stroke seem to crucially influence the long-term prognosis of the disease.

To current knowledge, it is unclear to what extent the above-mentioned preclinical findings can be transferred to a clinical setting. Also, based on our results one cannot differentiate a mere association (biomarker function) from a causal relationship (pathomechanism) between MV and cardiovascular outcome. Both interpretations are possible in this context and likely influence our findings.

4.2 Limitations

When interpreting the present study results, several limitations should be considered. Blood samples were taken within 7 days after stroke. Given the results from the pilot study, we must assume that differences in clearance rate cause fluctuations of MV levels over short time intervals.[46] Thus, not standardizing the exact time point of blood draw remains a flaw in our study design. To date, the exact behavior of MV levels during the first week after stroke has not been studied in detail, so that the extent to which this might influence our results is unknown. We accounted for this issue by treating the day of blood draw after stroke as a confounder and adjusting for it in the survival analysis. By excluding severe strokes, our results only apply to mildly to moderately affected patients so that external validity is impaired. Besides, we chose to set the cut-offs in our analysis at 75^{th} percentile and grouped in quartiles. This

selection of cut-offs was exploratory and does not reflect a biological function. In previous studies, cut-offs have also been chosen artificially (median, quartiles) and evidence for these needs yet to be established. Furthermore, due to legal constraints we could not access the death certificates of the patients so that the etiology of death could not be determined.

4.3 Outlook, Clinical perspective, Future Research

The present findings provide some in-depth information about possible underlying pathomechanims in the long-term cardiovascular outcome after stroke. Future research should validate these with even stricter standardized protocols regarding the time point of blood draw. Also, repeated MV measurements would be valuable to assess the changes of MV levels over time, as our pilot study suggests that they are a highly dynamic biomarker. We are currently investigating MV with the same methodology in another study with stroke patients (Biomarkers and perfusion – training-induced changes after stroke, BAPTISe)[47], which includes repeated measurements and can provide more information about the behavior of MV levels over time. Also, this is a more severely affected cohort, estimating if the present findings can be generalized to severe strokes.

In a clinical context, the detection of MV levels as biomarkers for cardiovascular risk after stroke is a very promising perspective. They might be useful to identify subgroups with high risk of a recurrent cardiovascular event or death from a cohort of stroke patients. Preventive strategies aimed at controlling classical risk factors associated with MV such as arterial hypertension could have a beneficial effect both on lowering MV levels and clinical outcome. Furthermore, MV might serve as parameter for monitoring of therapeutic effect. In context of our study group, our findings might add predictive value to existing classical cardiovascular risk factors, for example in a receiver operating characteristic (ROC) curve. The prediction model with the validation cohort PROSCIS-M is currently under investigation.

However, before MV measurements can be implemented in clinical routine, their detection systems have to be standardized and facilitated. As explained in the limitations, the current methodology for MV detection is not sufficiently comparable between centers. Besides, the techniques used are too elaborate for large-scale clinical practice.

Until MV can be widely used in a clinical setting, further understanding their pathophysiological role in stroke remains a main interest. For instance, it is currently unknown, how blood-brain barrier disruption in stroke influences MV exchange between peripheral blood and the central nervous system. We are planning to investigate the relationship between MV and stroke lesion volume in our cohort which could shed some light on this question. Linking EMV levels with other parameters of endothelial dysfunction such as ankle-brachial index could reinforce their causal role. Further projects involve exploration of the relationship between LMV and other biomarkers of inflammation such as hs-CRP, and blood-leukocyte levels. This might enhance understanding of the proposed underlying pathomechanisms by which EMV and LMV influence the disease in a clinical patient setting.

Based on the assumption of a causal relationship, EMV and LMV might be targets for future therapies to improve long-term outcome after stroke.

4.4 Conclusion

To conclude, our study demonstrated that high levels of circulating EMV and LMV in the acute phase after mild to moderate ischemic stroke are associated with a higher rate of secondary cardiovascular events and deaths during the 3-year long-term follow-up. These findings suggest that endothelial dysfunction and vascular inflammation might significantly impact the long-term prognosis and cardiovascular risk prediction of the disease.

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6 Affidavit

I, Shufan Huo, by personal signature in lieu of an oath, hereby affirm that I have written the submitted dissertation with the topic:

"Circulating Microvesicles and Cardiovascular Risk after Ischemic Stroke

Zirkulierende Mikrovesikel und kardiovaskuläres Risiko nach ischämischem Schlaganfall"

independently and without undisclosed help from third parties and have used no sources and resources other than those specified. All passages that are based literally or contextually on publications or lectures by other authors are identified as such in the correct citation. I am responsible for the sections on methodology (especially practical work, laboratory experiments, statistical processing) and results (especially figures, graphics and tables).

I also assure that I have correctly identified the data, data analyses and conclusions generated in cooperation with other people and correctly identified my own contribution as well as the contributions of others (see statement of contribution). No texts or parts of text were created or processed jointly with others.

My contributions to any publications on this dissertation correspond to those specified in the joint declaration below with the first supervisor. The guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.og) on authorship were complied with for all publications produced in the context of the dissertation. I also declare that I commit to comply with the regulations of Charité - Universitätsmedizin Berlin ensuring good scientific practice.

I also assure that I have not submitted this dissertation to any other faculty in the same or a similar form.

I am aware of the importance of this affidavit and the criminal consequences of an untrue affidavit (§§156, 161 of the German Criminal Code).

Date Signature

7 Statement of Contribution

The above work is based upon the publication:

Shufan Huo, Nicolle Kränkel, Alexander H. Nave, Pia S. Sperber, Jessica L. Rohmann, Sophie K. Piper, Peter Heuschmann, Ulf Landmesser, Matthias Endres, Bob Siegerink, Thomas G. Liman. Endothelial and leukocyte-derived microvesicles and cardiovascular risk after stroke – PROSCIS-B. *Neurology.* Nov 2020. doi: 10.1212/WNL.0000000000011223.

I hereby confirm that I, Shufan Huo, have contributed to this publication in the following detail:

From the beginning of this project, I have participated in developing the final research question. In this context, I have drafted the detailed analysis plan including the pilot analysis. For the experimental part of the project, I have developed the protocol and performed all flow cytometry measurements of microvesicles and gating procedures. I wrote all scripts for data import, data cleaning, transformation into the final dataset, endpoint formation, statistical analyses and generation of all tables and figures. Furthermore, I have participated in endpoint validation by hospital records. Finally, I have drafted, revised and submitted the present manuscript as first and corresponding author.

Date and signature of supervisor (Dr. med. Thomas G. Liman)

Date and signature of doctoral candidate (Shufan Huo)

8 Extract from the Journal Summary List (ISI Web of KnowledgeSM)

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"CLINICAL NEUROLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 199 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	LANCET NEUROLOGY	30,748	28.755	0.069460
2	Nature Reviews Neurology	9,548	21.155	0.031060
3	ACTA NEUROPATHOLOGICA	20,206	18.174	0.041660
4	Alzheimers & Dementia	13,341	14.423	0.036340
5	JAMA Neurology	8,683	12.321	0.042040
6	BRAIN	52,970	11.814	0.074030
7	SLEEP MEDICINE REVIEWS	6,920	10.517	0.010920
8	NEURO-ONCOLOGY	11,858	10.091	0.029150
9	ANNALS OF NEUROLOGY	37,336	9.496	0.048630
10	NEUROLOGY	89,258	8.689	0.115200
11	JOURNAL OF NEUROLOGY NEUROSURGERY AND PSYCHIATRY	29,660	8.272	0.030730
12	MOVEMENT DISORDERS	26,964	8.061	0.037650
13	Neurology-Neuroimmunology & Neuroinflammation	1,996	7.353	0.008220
14	Brain Stimulation	5,457	6.919	0.014470
15	Epilepsy Currents	799	6.909	0.001560
16	NEUROPATHOLOGY AND APPLIED NEUROBIOLOGY	3,876	6.878	0.006420
17	NEUROSCIENTIST	4,986	6.791	0.008520
18	BRAIN PATHOLOGY	5,263	6.155	0.007880

9 Publication

S. Huo, N. Kränkel, A. H. Nave, P. S. Sperber, J. L. Rohmann, S. K. Piper, U. Landmesser, M. Endres, and B. Siegerink, Endothelial and leukocyte-derived microvesicles and cardiovascular risk after stroke – PROSCIS-B. *Neurology*. Nov 2020.

doi: 10.1212/WNL.0000000000011223

10 Curriculum Vitae

Due to reasons of data protection, my curriculum vitae is not published in the electronic version of my thesis.

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11 List of Publications

S. Huo, N. Kränkel, A. H. Nave, P. S. Sperber, J. L. Rohmann, S. K. Piper, U. Landmesser, M. Endres, and B. Siegerink, Endothelial and leukocyte-derived microvesicles and cardiovascular risk after stroke – PROSCIS-B. *Neurology.* Nov 2020. doi: 10.1212/WNL.0000000000011223. Online ahead of print. Impact Factor 8.689

P. S. Sperber, B. Siegerink, J. L. Rohmann, **S. Huo**, S. K. Piper, H. Prüß, P. Heuschmann, T. G. Liman*, M. Endres* (*equal contribution). Serum Anti-NMDA (N-Methyl-D-Aspartate)-Receptor Antibodies and Long-Term Clinical Outcome After Stroke (PROSCIS-B). *Stroke.* Nov 2019. 50(11):3213-3219. doi: 10.1161/STROKEAHA.119.026100. Impact Factor 7.190

J. L. Rohmann, **S. Huo**, P. S. Sperber, S. K. Piper, F. R. Rosendaal, P. U. Heuschmann, M. Endres, T. G. Liman, B. Siegerink. Coagulation factor XII, XI, and VIII activity levels and secondary events after first ischemic stroke. *J Thromb Haemost.* Sep 16 2020. doi: 10.1111/jth.15092. Online ahead of print. Impact Factor 4.157

S. Major, **S. Huo**, C. L. Lemale, E. Siebert, D. Milakara, J. Woitzik, K. Gertz, J. P. Dreier. Direct electrophysiological evidence that spreading depolarization-induced spreading depression is the pathophysiological correlate of the migraine aura and a review of the spreading depolarization continuum of acute neuronal mass injury. *Geroscience.* Feb 2020;42(1):57-80. doi: 10.1007/s11357-019-00142-7. Impact Factor 4.360

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