Endothelial and Leukocyte-Derived Microvesicles and Cardiovascular Risk After Stroke

PROSCIS-B

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Abstract

Objective
To determine the role of circulating microvesicles (MV) on long-term cardiovascular outcomes after stroke, we measured them in patients with first-ever stroke with a 3-year follow-up.

Methods
In the Prospective Cohort With Incident Stroke Berlin (PROSCIS-B), patients with first-ever ischemic stroke were followed up for 3 years. The primary combined endpoint consisted of recurrent stroke, myocardial infarction, and all-cause mortality. Citrate-blood levels of endothelial MV (EMV), leukocyte-derived MV (LMV), monocytic MV (MMV), and platelet-derived MV (PMV) were measured with flow cytometry. Kaplan-Meier curves and adjusted Cox proportional hazards models were used to estimate the effect of MV levels on the combined endpoint.

Results
Five hundred seventy-one patients were recruited (median age 69 years, 39% female, median NIH Stroke Scale score 2, interquartile range 1–4), and 95 endpoints occurred. Patients with levels of EMV (adjusted 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 highest quartile were more likely to experience an event than participants with lower levels with the lowest quartile used as the reference category. The association was less pronounced for PMV (HR 1.7, 95% CI 0.9–3.2) and absent for MMV (HR 1.1, 95% CI 0.6–1.8).

Conclusion
High levels of EMV and LMV after stroke were associated with worse cardiovascular outcome within 3 years. These results reinforce that endothelial dysfunction and vascular inflammation affect the long-term prognosis after stroke. EMV and LMV might play a role in risk prediction for stroke patients.

ClinicalTrials.gov Identifier
NCT01363856.

Classification of Evidence
This study provides Class II evidence of the effect of MV levels on subsequent stroke, myocardial infarction, or all-cause mortality in survivors of mild stroke.
Endothelial dysfunction (ED) is characterized by a proinflammatory and procoagulant state with impaired vascular reactivity. It is an early component of atherosclerosis and an established risk factor for poor cardiovascular outcome.\textsuperscript{2–5} Circulating endothelial microvesicles (EMV) are novel blood-based biomarkers for ED.\textsuperscript{6,7} Microvesicles (MV) are small membrane vesicles (<1 μm) shed by cells in response to inflammatory activation. According to the European Society of Cardiology Working Group on Atherosclerosis and Vascular Biology, MV are thought to have great potential as novel biomarkers in cardiovascular risk stratification.\textsuperscript{8} In cardiovascular medicine, EMV and immune cell-derived extracellular vesicles such as leukocyte-derived MV (LMV) are thought to contribute to different stages of disease development, for example, in atherosclerosis by promoting ED, unstable plaque progression, and thrombus formation.\textsuperscript{9} In the Framingham Heart Study, EMV levels were associated with atherosclerosis progression and correlated with cardiometabolic risk factors.\textsuperscript{10} Furthermore, EMV levels are associated with poor long-term cardiovascular outcome in patients with stable coronary artery disease (CAD) and with lesion size after acute myocardial infarction (MI).\textsuperscript{11–14} In neurologic diseases such as Alzheimer disease, multiple sclerosis, and traumatic brain injury, there has also been research showing increased EMV levels compared to healthy controls and associations of EMV levels with disease severity and unfavorable clinical outcome.\textsuperscript{15} In ischemic stroke, EMV levels are elevated and correlate with stroke severity and lesion volume.\textsuperscript{16,17} Levels of MV of other origin such as LMV, monocyteic MV (MMV), and platelet-derived MV (PMV) are also increased in the acute phase after ischemic stroke.\textsuperscript{18,19} Knowledge about the role of increased levels of circulating EMV and MV of other origins in long-term prognosis after stroke remains scarce.

In this study, we investigated an association of MV of endothelial, leukocyte-derived, monocyteic, and platelet-derived origin with a combined cardiovascular outcome in a large prospective cohort of patients with first-ever stroke followed up for 3 years.

Methods

**Standard Protocol Approvals, Registrations, and Patient Consents**

Patients or their legal representatives gave written informed consent before study participation. The study protocol was approved by the ethics committee (internal review board) of the Charité-Universitätsmedizin Berlin (EA1/218/09) and was conducted in accordance with ethical principles described in the Declaration of Helsinki.

**The PROSCIS-B Study**

The Prospective Cohort With Incident Stroke Berlin (PROSCIS-B, ClinicalTrials.gov identifier: NCT01363856) is a prospective, observational, hospital-based cohort study of patients enrolled after first-ever stroke. The previously published study protocol and biomarker analysis provide details.\textsuperscript{20,21} In short, patients with ischemic stroke, primary hemorrhage, or sinus venous thrombosis were recruited between January 2010 and June 2013 at 1 of 3 stroke units of Charité-Universitätsmedizin Berlin. Within 7 days of stroke onset, patients received interviews, extensive clinical evaluations, and blood draws for laboratory analysis.\textsuperscript{20} During 3 years of follow-up, annual telephone-based interviews assessed patients’ vital status, incidence of cardiovascular diseases, and functional outcome. Vital status was additionally obtained from the local registry office, even if lost to follow-up.

**Study Population**

Patients ≥18 years of age were included after first-ever stroke as defined by World Health Organization criteria.\textsuperscript{22} Exclusion criteria were previous stroke (not counting TIA\textsuperscript{22}), brain tumor or metastases, and participation in any intervention study. Due to limited recruitment of severely affected patients, only patients with mild to moderate ischemic stroke (NIH Stroke Scale [NIHSS] score ≤15) were included in this analysis.

**Patient Characteristics**

Additional information at baseline was collected, including sociodemographic parameters (age and sex), etiologic subtype of stroke according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification,\textsuperscript{23} stroke severity according to NIHSS score (mild 0–4, moderate 5–15),\textsuperscript{24} cardiovascular risk factors (current smoking, alcohol consumption, hypertension, peripheral artery disease, prevalent atrial fibrillation, prevalent diabetes mellitus, history of MI), thrombolysis, and number of days between stroke and blood draw.

**Primary Endpoint**

Our main outcome is a predefined combined cardiovascular endpoint consisting of the first occurrence of recurrent ischemic or hemorrhagic stroke, MI, or all-cause death within 3
years. In 1 case, the date of death was not provided and was set halfway between last contact with patient and time stamp of the returned questionnaire. All endpoints were confirmed by medical records from the treating physician. An endpoint committee consisting of 2 senior vascular neurologists not involved in the study independently rated and validated the endpoints according to the World Health Organization criteria.22

Deaths were confirmed by the local registration office, and additional unreported endpoints were identified through the Charité University Hospital medical records.

**Blood Sample Collection and Storage**

Citrate-buffered blood (2.7 mL) was collected after an overnight fast during days 1 through 7 after initial presentation. Within 2 hours, a 2-step centrifugation protocol at room temperature was then used to retrieve platelet-depleted plasma (1,500× relative centrifugal force [rcf], 5 minutes; 13,500× rcf, 5 minutes) before storage at −80°C until measurement.25

**Sample Staining and Flow Cytometry**

Fluorochrome-coupled antibodies for MV phenotyping were combined and centrifuged (16,000× rcf, 20 minutes, 4°C) before sample staining to minimize antibody aggregates. Thawed plasma was centrifuged (1,500× rcf, 10 minutes, 4°C) to eliminate larger particles and then incubated with antibodies for 20 minutes in the dark at room temperature.

MV were measured by flow cytometry with an Attune Nxt acoustic focusing cytometer (Thermo Fisher Scientific, Waltham, MA) with red (637 nm), blue (488 nm), and violet (405 nm) lasers. Gating was performed with Kaluza software (version 1.5; Beckman & Coulter, Brea, CA). Size cutoff of 1 μm was set with fluorescent beads (Megamix-Plus SSC; BioCytek, Marseille, France). All MV were defined as annexin V (AV)–binding particles <1 μm. We have tested the lower limit of resolution of the Attune Nxt acoustic focusing cytometer with beads in sizes 20, 100, 300, 500, 1,000, and 2,000 nm (Flow Cytometry Sub-micron Particle Size Reference Kit; Thermo Fisher Scientific). Whereas the 20-nm bead populations were not visible, all other beads could be clearly distinguished, rendering a lower detection limit of about 100 nm suitable for MV measurement. Sequential gating was applied according to established cell-specific surface markers to define individual populations (figure 1 and table 1) and data available from Dryad, table e-1: doi:10.5061/dryad.q2bqv83gn).

Fluorescence-minus-one controls were used for discrimination from background. Lipid-membrane vesicle identity of gated events was confirmed with the detergent Triton X-100 (final concentration 0.1%). After treatment with Triton X-100, the AV-positive MV population from the same sample was no longer visible in flow cytometry. MV from the supernatant of cultured human aortic endothelial cells (HAEC, Lonza, Basel, Switzerland), induced pluripotent stem cell-derived endothelial cells, and human peripheral blood mononuclear cells were used as positive controls. Acquisition and gating were performed by investigators blinded to patient characteristics and outcomes.

**Statistical Analysis**

All statistical analyses except the sensitivity and post hoc analyses were predefined in an analysis plan before data acquisition to which the authors adhered at all times.

Patients were categorized into quartiles by MV count. Event-free survival function was estimated with the Kaplan-Meier technique with quartile 1 as a reference category. The log-rank test was used to assess difference in survivorship between the groups. Person-time was defined as the time between initial ischemic stroke event and the combined cardiovascular endpoint or time of censorship, whichever occurred first. Participants were censored at time of last contact or at 3 years of follow-up.

In a secondary analysis, patients were dichotomized using the 75th percentile of MV count as the cutoff. This cutoff was chosen a priori to test our hypothesis that high levels of MV are related to large acute tissue damage (EMV), coagulation activity (PMV), or acute inflammatory response (LMV, MMV), whereas low variations can be influenced by multiple causes.

Differences in event rates were then quantified with hazard ratios (HRs) and corresponding 95% confidence intervals (CIs) from Cox proportional hazards regression models. Adjustment for confounding was performed in 3 iterative models: model 1, adjusted for age (continuous), sex, and NIHSS score (mild 0–4, moderate 5–15); model 2, additionally adjusted for TOAST classification (categorical)23 and dichotomous cardiovascular risk factors (current smoking, alcohol consumption, hypertension, peripheral artery disease, atrial fibrillation, diabetes mellitus, MI); and model 3, additionally adjusted for thrombolysis (yes/no) and number of days between stroke and blood draw.

In our gating strategy, the different entities of MV types were defined excluding one another to avoid overlap (figure 1 and table 1). First, PMV were defined. From the remaining particles, LMV and MMV were identified, and subsequently, EMV were defined as particles that are neither PMV nor LMV/MMV. Thus, the measurements of the different MV subpopulations are dependent on each other, especially because it is not possible to clearly distinguish between them due to population overlap in flow cytometry (figure 1). This is a technical limitation, due mainly to the fact that flow cytometry is optimized for larger particles such as cells. Nonetheless, this means that the values of the different MV types depend on each other, and therefore, the presence of one type may confound the relationship under study between another type
and the outcome. In a sensitivity analysis, we additionally adjusted for the other MV types, specifically LMV and MMV for PMV and EMV for LMV. The MV variables were included in the model as continuous covariates after logarithmic transformation for normal distribution.

Because elevated EMV and LMV levels are related to ED and vascular inflammation, we have performed an additional posthoc interaction analysis between EMV/LMV and large artery atherosclerotic (LAA) strokes. Due to low numbers in some joint exposure categories, this analysis was adjusted only for age and sex.

All analyses were performed with STATA/IC 14.2 (Stata Statistical Software, release 14; StataCorp LP, College Station, TX, 2015). All clinical data and analysis codes are stored at the Center for Stroke Research, Charité-Universitätsmedizin Berlin, Germany.

S. Huo, T.G. Liman, and B. Siegerink take full responsibility for the clinical data, the integrity of its analyses and interpretation, and the conduct of the research and have full access to all primary data and the right to publish any and all data.

Classification of Evidence
This study provides Class II evidence of the impact of MV levels on subsequent stroke, MI, or all-cause mortality in survivors of mild stroke.

Data Availability
Anonymized data will be shared on reasonable request from any qualified investigator.

Results
Between January 2010 and June 2013, 690 patients were recruited for PROSCIS-B, of whom 21 withdrew consent after inclusion. Of the remaining 669 patients, 42 had either hemorrhagic strokes or sinus venous thromboses. Of the 627 patients with ischemic stroke, only 6 were severely affected.

### Table 1 Surface Markers Defining MV Populations in Flow Cytometry

| Original Cell | Surface Markers                              |
|---------------|----------------------------------------------|
| All MV        | AV+                                          |
| PMV           | AV+ and CD41+                                |
| LMV           | AV+ and CD41− and CD45+                      |
| MMV           | AV+ and CD41− and CD14+                      |
| EMV           | AV+ and CD41− and CD45− and CD31+ or CD144+ or CD146+ |

Abbreviations: AV = annexin V; EMV = endothelial MV; LMV = leukocyte-derived MV; MMV = monocytic MV; MV = microvesicles; PMV = platelet-derived MV. EMV were defined as carrying ≥1 of 3 surface markers characteristic for EMV.
(NIHSS score ≥16) and therefore excluded from the analysis. Ultimately, 571 of these eligible individuals (92%) had data on MV measurements and were included in our analyses (figure 2).

Baseline characteristics of the cohort including MV measurements are shown in table 2. We additionally provide an overview of baseline characteristics stratified by quartiles of EMV and LMV (data available from Dryad, table e-2: doi:10.5061/dryad.q2bq83gn) and stratified by occurrence of the combined endpoint (data available from Dryad, table e-3). Median age at baseline was 69 years (interquartile range [IQR] 58–76 years), with 39% female and a median NIHSS score of 2 (IQR 1–4). Median day of blood draw after stroke was 4 (IQR 3–5). Among all types of MV, PMV demonstrated the highest median concentrations measured in plasma (1.27/μL, IQR 0.57–2.69/μL). EMV (0.26/μL, IQR 0.13–0.54/μL) and LMV (0.29/μL, IQR 0.15–0.66/μL) median levels were lower at baseline. Median MMV plasma concentrations were the lowest (0.001/μL, IQR 0–0.002/μL). After natural logarithmic transformation, MV measurement data were normally distributed. MV levels were not related to stroke severity measured by NIHSS score in our sample. During follow-up of the 571 persons at risk, 95 combined cardiovascular endpoint events were recorded, including 42 ischemic or hemorrhagic stroke recurrences, 5 MIs, and 48 all-cause deaths. The total person-time was 1,407 person-years (py), resulting in an incidence rate of 67.5 combined endpoint events per 1,000 py.

Kaplan-Meier event-free survivorship curves stratified by MV quartiles are shown in figure 3. No visible violations of the proportional hazards assumption were observed, especially in comparison of quartiles 1 and 4 and high (≥75th percentile) vs low (<75th percentile). Differences in the crude comparison of the curves were observed between quartile groups of LMV ($p = 0.01$). No significant differences between quartile groups were found for EMV ($p = 0.06$), PMV ($p = 0.38$), and MMV ($p = 0.44$).

For the Cox proportional hazards regression, HR and CI for quartiles of MV for the fully adjusted model 3 are listed in table 3 with the lowest quartile as reference category. The observed adjusted HR for the quartile 4 versus 1 comparison was 2.5 (95% CI 1.2–4.9) for EMV, 3.1 (95% CI 1.4–6.8) for LMV, 1.7 (95% CI 0.9–3.2) for PMV, and 1.1 (95% CI 0.6–1.8) for MMV. Data available from Dryad (tables e-4.1–e-4.4: doi:10.5061/dryad.q2bq83gn) give a detailed overview of HRs from the crude model and models 1 through 3 of MV quartiles.

Secondary analyses for MV group-specific incidence rates and HRs comparing high (≥75th percentile) and lower (<75th percentile) levels are shown in table 4. The HR for the combined endpoint in the fully adjusted Cox proportional hazards regression (model 3) was 1.6 (95% CI 1.0–2.4) for EMV, 1.5 (95% CI 1.0–2.4) for LMV, 1.4 (95% CI 0.9–2.2) for PMV, and 1.3 (95% CI 0.7–2.4) for MMV.

In a sensitivity analysis, we estimated the effects of each MV type conditional on the other MV types by adding these as covariables. After adjustment of LMV and MMV for PMV and adjustment of EMV for LMV according to the gating procedure, the HR remained unchanged. Table 4 provides details.

Data available from Dryad (tables e-5.1 and e-5.2: doi:10.5061/dryad.q2bq83gn) give an overview of the results of our interaction analysis between EMV/LMV and LAA. Patients with high EMV levels and LAA showed a 1.8-fold hazard (95% CI 0.9–3.7) compared to those with neither high EMV levels nor LAA. Patients with high LMV levels and LAA showed a
Table 2 Baseline Characteristics

| Baseline Characteristics | PROSCIS-B (n = 621) |
|-------------------------|---------------------|
| Age, median, IQR, y     | 69, 58–76           |
| Female sex, n, %        | 242, 39             |
| NIHSS score, median, IQR| 2, 1–4              |
| 0–4, n, %               | 470, 76             |
| 5–15, n, %              | 151, 24             |
| TOAST, n, %             |                     |
| Large artery atherosclerosis | 167, 27          |
| Cardioembolic           | 145, 23             |
| Small artery occlusion  | 96, 15              |
| Other causes            | 22, 4               |
| Undefined               | 191, 31             |
| Cardiovascular risk factors, n, % |                     |
| History of myocardial infarction | 21, 3   |
| Diabetes mellitus       | 137, 22             |
| Atrial fibrillation     | 132, 21             |
| Peripheral artery disease| 42, 7             |
| Arterial hypertension   | 406, 65             |
| Current smokers         | 171, 28             |
| Alcohol consumption     | 217, 35             |
| Therapy                 |                     |
| Thrombolysis, n, %      | 125, 20             |
| Day of blood draw after stroke, median, IQR | 4, 3–5 |
| Patients with analysis of microvesicle count (all subtypes), n | 517 |
| Microvesicle concentrations, median, IQR, μL⁻¹ |                     |
| Endothelial             | 0.26, 0.13–0.54     |
| Leukocyte-derived       | 0.29, 0.15–0.66     |
| Platelet-derived        | 1.27, 0.57–2.69     |
| Monocytic               | 0.001, 0–0.002      |

Abbreviations: IQR = interquartile range; NIHSS = NIH Stroke Scale; PROSCIS-B = Prospective Cohort with Incident Stroke study Berlin; TOAST = Trial of Org 10,172 in Acute Stroke Treatment Classification.

1.5-fold hazard (95% CI 0.7–3.1) compared to those with neither risk factor. These increases in risk for those with both exposures seem to be in line with the sum of the effect estimates for each separate risk factor. After adjustment for age and sex, the effect is less pronounced for EMV and no longer seen for LMV. However, due to the relatively low number of patients in some of these joint exposure categories, we caution against overinterpretation of these results because they are not very precise (large CI) and some residual confounding is likely.

Discussion

Our study showed that higher plasma levels of LMV (defined as AV+ CD45+ CD41−) and EMV (defined as AV+ CD31+ /CD144+/CD146+ CD41− CD45−) were significantly associated with an increased risk for a combined cardiovascular endpoint including MI, recurrent stroke, or death over an observational period of 3 years after first ischemic stroke. No clear association was observed for PMV (defined as AV+ CD41+) and MMV (defined as AV+ CD41– CD14+) levels in our prospective cohort study.

So far, most clinical studies on MV originate from the field of cardiology, whereas data linking MV and outcome after stroke are limited. Two previous studies investigated the significance of EMV as a biomarker for future cardiovascular diseases in patients with CAD. One group found that high levels of CD31+ AV+ MV are associated with a higher risk of cardiovascular events in 200 patients with stable CAD over a median follow-up time of 6.1 years. Another study with patients from a high-risk population for CAD showed that increased CD144+ MV levels are independent predictors for cardiovascular death and acute coronary syndrome. Others demonstrated that MV levels are increased in the acute phase of MI and associated with disease severity and mortality.

While MI and ischemic stroke share most of their common risk factors, major differences exist concerning etiology, treatment options, and regenerative potential of the damaged tissue. Thus, corresponding to the findings in MI, research on MV as biomarkers in stroke as a more heterogeneous disease is growing. Thirteen case-control studies have compared MV levels in patients with ischemic stroke to MV levels in healthy controls as summarized by a recent systematic review. Overall, these studies demonstrated that MV levels of several origins such as EMV, LMV, PMV, and MMV are elevated after stroke. Elevated LMV levels of CD45+, CD14+, CD4+, and CD15+ were observed after acute ischemic stroke, whereas only CD14+ levels were associated with stroke severity. Furthermore, one study found an association of higher CD62E+ MV levels with higher NIHSS scores and larger infarct volumes in acute stroke.

In the chronic course after stroke, one study investigated the association of increased EMV levels on cardiovascular outcomes. This group measured levels of CD62E+ and CD31+ AV+ MV in 298 patients ≥3 months after stroke and followed them for 36 months. Among 29 patients with major cardiovascular events, including 13 recurrent ischemic strokes, higher CD62E+ MV levels were associated with cardiovascular events, whereas high CD31+ AV+ levels were not. Our study extends these findings in a larger cohort and adds MV of other origins. Moreover, we used a more specific phenotyping to detect MV of endothelial
origin (AV+ CD31+/CD144+/CD146− CD41− CD45−); that is, we could differentiate nonendothelial MV of platelet-derived (CD41+) origin given that CD31 binds to both EMV and PMV. However, methods for isolating and detecting MV in clinical studies widely vary (e.g., sample preparation, flow cytometry protocols, cell-surface phenotyping), making it difficult to compare the joint evidence from these studies. The European Society of Cardiology and the International Society for Extracellular Vesicles therefore strive for standardization of MV measurement protocols and published recent consensus documents in 2018. Our methodology is based on these recommendations.

Physiologically, there is mounting evidence that MV play an important regulatory role particularly in vascular diseases by transporting regulatory molecules, for example, RNAs, lipids, and proteins. In vitro, EMV could promote inflammation, endothelial activation, vascular dysfunction, coagulation, and progression of atherosclerosis. In addition, EMV and LMV impair the endothelium-dependent relaxation by disturbing the endothelial nitric oxide transduction and prostacyclin pathways and induce prothrombotic activity via a tissue factor/factor VII–dependent pathway. LMV as proinflammatory mediators might also contribute to vascular inflammation. Thus, ED and a proinflammatory and prothrombotic state might be present in patients with stroke with increased EMV and LMV levels, leading to the increased long-term risk of adverse cardiovascular outcome. However, it is unclear whether these possible underlying pathomechanisms from in vitro studies can be transferred to a clinical setting. On the basis of our observational cohort, we cannot differentiate an association (biomarker) from causation (biological pathomechanism) between MV and cardiovascular outcome; both are possible explanations and likely play a role in our results.

PMV levels showed no association with vascular risk in our study. PMV are considered biomarkers of thrombotic state and platelet activation. Thus, one could assume that higher

**Figure 3** Kaplan-Meier Cumulative Probability of Combined Cardiovascular Endpoint Curves for Quartile Groups of MV Levels

Crude cumulative probability curves for each quartile of microvesicle (MV) levels are displayed. Quartile (Q) 4 of MV levels had the highest cumulative probability of events of all analyzed MV phenotypes, while Q1 had the lowest cumulative event probability for endothelial MV (EMV), leukocyte-derived MV (LMV), and platelet-derived MV (PMV). No clear group differences were observed for monocytic MV (MMV). The p values result from the log-rank test.
PMV levels might be associated with recurrent vascular events after stroke. However, PMV measurement is most vulnerable to technical inaccuracy because, despite per-protocol centrifugation cycles, platelets may be incompletely depleted from samples, causing misinterpretation.

Several limitations have to be considered in this prospective stroke cohort. First, blood was taken within 7 days after the index event and thus during the acute phase. We need to take into account that differences in clearance rates of MV lead to fluctuations of MV levels over time, something we are not able to quantify with our single blood draw. However, we tried to incorporate this by adjusting in model 3 for the number of days between stroke and blood draw. Furthermore, we limited our analysis to only mild to moderate ischemic strokes (NIHSS score 0–15, excluding 6 patients with NIHSS ≥16) to increase the homogeneity of the cohort and to reduce sampling bias. This impedes the generalizability of our results to patients with severe stroke. Moreover, we chose the quartile and 75th percentile cutoffs for a high vs low comparison, which do not reflect a biological step function because evidence for this is lacking. In addition, the use of quartiles may limit comparability to other studies. Furthermore, due to legal restrictions, we had no access to the death certificates, so we could not determine the cause of the deaths.

Our study shows that high levels of EMV and LMV in the acute phase after ischemic stroke are associated with increased long-term risk of cardiovascular events and all-cause mortality. These results suggest that ED and vascular inflammation might play a causal role in the long-term prognosis after ischemic stroke. However, future studies

### Table 3 Adjusted Cox Proportional Hazards Regression Analysis for Quartiles of MV Subtype

| MV Levels in Quartile Groups | HR, EMV 95% CI | HR, LMV 95% CI | HR, PMV 95% CI | HR, MMV 95% CI |
|-----------------------------|----------------|----------------|----------------|----------------|
| Q1 (reference)              | 1              | 1              | 1              | 1              |
| Q2                          | 1.9            | 0.9–4.0        | 3.7            | 1.7–8.2        | 1.4            | 0.7–2.8        | 0.8            | 0.4–1.6        |
| Q3                          | 1.7            | 0.8–3.5        | 1.7            | 0.7–3.9        | 1.3            | 0.7–2.5        | 1.0            | 0.5–1.8        |
| Q4                          | 2.5            | 1.2–4.9        | 3.1            | 1.4–6.8        | 1.7            | 0.9–3.2        | 1.1            | 0.6–1.8        |

Abbreviations: CI = confidence interval; EMV = endothelial MV; HR = hazard ratio; LMV = leukocyte-derived MV; MMV = monocytic MV; MV = microvesicles; PMV = platelet-derived MV; Q = quartile.

HRs and 95% CI limits were estimated by Cox proportional hazards regression models and adjusted for confounding according to model 3 (age, sex, NIH Stroke Scale score, Trial of Org 10,172 in Acute Stroke Treatment Classification [TOAST] classification, cardiovascular risk factors [current smoking, alcohol consumption, hypertension, peripheral artery disease, atrial fibrillation, diabetes mellitus, myocardial infarction], thrombolysis, and number of days between stroke and blood draw). For a detailed overview of the crude model and models 1 through 3, see tables e-4.1 through e-4.4 available from Dryad (doi:10.5061/dryad.q2bvq83gn).

### Table 4 MV Group-specific IR and HR Comparing High (≥75th Percentile) vs Lower Levels (<75th Percentile)

| MV type | p75 (μL⁻¹) | No. | Events, n | IR, 1,000 py⁻¹ | HR0 (95% CI) | HR1 (95% CI) | HR2 (95% CI) | HR3 (95% CI) | HR4 (95% CI) |
|---------|------------|-----|-----------|----------------|---------------|---------------|---------------|---------------|---------------|
| EMV     | 0.54       |     |           |                |               |               |               |               |               |
|         | <p75       | 428 | 64        | 1,072.2        | 59.7          | 1.52          | 1.43          | 1.45          | 1.55          | 1.45          | (0.99–2.33)   | (0.93–2.20)   | (0.93–2.28)   | (0.99–2.45)   | (0.79–2.66)   |
|         | ≥p75       | 143 | 31        | 335.0          | 92.5          |               |               |               |               |               |
| LMV     | 0.66       |     |           |                |               | 1.63          | 1.37          | 1.53          | 1.53          | 1.40          | (1.06–2.49)   | (0.89–2.12)   | (0.97–2.39)   | (0.97–2.42)   | (0.83–2.35)   |
|         | <p75       | 428 | 62        | 1,059.3        | 58.5          |               |               |               |               |               |
|         | ≥p75       | 143 | 33        | 347.9          | 94.9          |               |               |               |               |               |
| PMV     | 2.69       |     |           |                |               | 1.26          | 1.25          | 1.34          | 1.39          | NA*          | (0.81–1.96)   | (0.80–1.95)   | (0.84–2.12)   | (0.87–2.21)   |
|         | <p75       | 428 | 67        | 1,055.4        | 63.5          |               |               |               |               |               |
|         | ≥p75       | 143 | 28        | 351.8          | 79.6          |               |               |               |               |               |
| MMV     | 0.002      |     |           |                |               | 1.40          | 1.19          | 1.22          | 1.22          | 1.14          | (0.91–2.14)   | (0.77–1.82)   | (0.78–1.90)   | (0.77–1.93)   | (0.71–1.82)   |
|         | <p75       | 416 | 63        | 1,029.2        | 61.2          |               |               |               |               |               |
|         | ≥p75       | 155 | 32        | 338.0          | 84.7          |               |               |               |               |               |

Abbreviations: CI = confidence interval; EMV = endothelial MV; HR = hazard ratio; IR = incidence rate; LMV = leukocyte-derived MV; MMV = monocytic MV; MV = microvesicles; NA = not applicable; PMV = platelet-derived MV; py = person-years; p75 = 75th percentile.

IR were calculated by dividing total number of combined endpoints by sum of person-time, reported per 1,000 py. HR and corresponding 95% CI were estimated in a series of adjusted Cox proportional hazards regression models: HR0, crude model; HR1, adjusted for age, sex, and NIH Stroke Scale score; HR2, additionally adjusted for Trial of Org 10,172 in Acute Stroke Treatment Classification (TOAST) and cardiovascular risk factors (current smoking, alcohol consumption, hypertension, peripheral artery disease, atrial fibrillation, diabetes mellitus, myocardial infarction); HR3, additionally adjusted for thrombolysis and number of days between stroke and blood draw; and HR4 (sensitivity analysis), EMV additionally adjusted for LMV and MMV/EMV additionally adjusted for PMV according to gating hierarchy.

* Gating of PMV occurred independently of other MV subtypes.
need to formally assess the added value of MV to existing risk prediction models. In addition, the underlying pathophysiologic mechanism by which MV affect poststroke outcome remains unclear, and further laboratory investigations are needed. MV that promote inflammation and ED may be future targets for possible therapies to improve prognosis after stroke.

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Disclosure
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| Sophie Käthe Piper, PhD | Charité-Universitätsmedizin, Berlin, Germany | Data analysis and revising manuscript |
| Peter Ulrich Heuschmann, MD | University of Würzburg, Germany | Study design, data acquisition |
| Ulf Landmesser, MD    | Charité-Universitätsmedizin, Berlin, Germany | Study design |
| Matthias Endres, MD   | Charité-Universitätsmedizin, Berlin, Germany | Study design, revising manuscript |
| Bob Siegerink, PhD    | Leiden University Medical Center, the Netherlands | Study design, data acquisition, analysis, and interpretation, review of biostatistical analysis, revising manuscript |
| Thomas Günter Liman, MD | Charité-Universitätsmedizin, Berlin, Germany | Study design, data acquisition, analysis, and interpretation, drafting and revising |
