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Circulating platelet-derived extracellular vesicles correlate with night-time blood pressure and vascular organ damage and may represent an integrative biomarker of vascular health

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Abstract

Elevated office blood pressure (BP) has previously been associated with increased levels of circulating extracellular vesicles (EVs). The present study aimed to assess the relationship between levels of platelet derived EVs, ambulatory BP parameters, and pulse wave velocity as a marker of macrovascular organ damage. A total of 96 participants were included in the study. Platelet-derived extracellular vesicles (pEVs) were evaluated by flow cytometry (CD41+/Annexin v+). BP evaluation included unobserved automated office BP and ambulatory BP monitoring. Carotid-femoral pulse wave velocity (PWV) was measured as a marker of macrovascular damage. pEVs correlated with nocturnal systolic BP (r = 0.31; p = .003) and nocturnal dipping (r = -0.29; p = .01) in univariable analysis. Multivariable regression models confirmed robustness of the association of EVs and nocturnal blood pressure (p = .02). In contrast, systolic office, 24h- and daytime-BP did not show significant associations with pEVs. No correlations were found with diastolic BP. Circulating pEVs correlated with pulse wave velocity (r = 0.25; p = .02). When comparing different hypertensive phenotypes, higher levels of EVs and PWV were evident in patients with sustained hypertension compared to patients with white coat HTN and healthy persons. Circulating platelet derived EVs were associated with nocturnal BP, dipping, and PWV. Given that average nocturnal BP is the strongest predictor of CV events, platelet derived EVs may serve as an integrative marker of vascular health, a proposition that requires testing in prospective clinical trials.

KEYWORDS
blood pressure, extracellular vesicles, thrombosis

1 | INTRODUCTION

Elevated blood pressure (BP) is an established risk factor for cardio-vascular events.[1–4] Hypertension (HTN) is a multifactorial condition and often characterized by a combination of chronic inflammation and shear stress promoting a pro-thrombotic state as demonstrated by the increased risk for major thrombotic complications such as stroke, myocardial infarction, and cardiovascular death.[5,6] Recently the ESC/ESH and ACC/AHA practice guidelines have updated their recommendations highlighting the importance of accurate blood pressure measurement in the diagnosis and management of HTN.[2,3] There is a consistent recommendation regarding the use of out-of-office BP measurements, either ambulatory blood pressure monitoring (ABPM) or home blood pressure monitoring (HBPM), to improve accuracy of BP measurements.[2,3,7–9] However, office blood pressure continues to be the cornerstone of clinical assessment as ABPM might not be available, is commonly associated with out of pocket expenses for patients, or may not be tolerated. ABPM is of particular importance for characterizing specific subpopulations that may otherwise remain undetected such as those with masked hypertension, nocturnal hyper-tension, or a non-dipping pattern. Each of these phenotypes have a strong association with cardiovascular events and hypertension mediated organ damage[2,3,8,9] at least in part mediated by altered thrombotic and fibrinolytic regulation.[10–12]

Extracellular vesicles (EVs) are small bilayer cell vesicles originating from the cell membrane in response to stress, injury, or cell activation.[13–16] There is growing evidence to suggest an association between EVs and cardiovascular disease.[15,17,18] Increased circulating EVs have been related to various clinical conditions impacting on vascular integrity, endothelial function, inflammation, and thrombosis, such as hypertension,[19–22] atherosclerosis,[17,18] heart failure,[23,24] and others.[25–28] EVs can originate from different cells including platelets, and their release can be promoted by coagulation, shear stress, hypoxia and proinflammatory mediators.[13,14,29,30] Consequently, it has been proposed that circulating EVs may reflect the overall status of vascular health by integrating the endothelial, thrombotic, and inflammatory status in an individual.[13,14,18] While some studies have demonstrated an association between blood pressure and endothelial EVs, the relation between BP and platelet derived extracellular vesicles (pEVs) has not yet been investigated in detail. The latter may be particularly important given that thrombotic events represent one of the most detrimental complications of hypertension.[2,5,31] The present analysis therefore focused on platelet derived EVs, as they have been suggested to be a marker of platelet activation.

The aim of the present study was to evaluate the relationship between circulating pEVs and BP levels assessed by office and out-of-office measurements (unobserved automated office BP, average 24 h BP, average day-time BP, and average night-time BP) and macrovascular organ damage assessed by pulse wave velocity.

2 | METHODS

2.1 | Participant population and study design

The study population consisted of a total of 100 participants presenting for diagnostic, workup, and clinical management of cardiovascular disease at the outpatient hypertension clinic of the Royal Perth Hospital. Patients who had heart failure NYHA class III-IV, chronic kidney disease (eGFR of < 30 mL/min/1.73 m^2) or active autoimmune disease requiring treatment with corticosteroids or other immunosuppressive agents were not eligible to be included in this analysis.

Reference group: In view of the absence of established reference values for EV, and as our main objective was not to compare between normotensive vs hypertensives but rather to explore the relationship between EV and blood pressure levels across the entire blood pressure spectrum (normotension, mild-, moderate- and severe-BP levels) a 10% of the sample was implemented as a reference group (10 younger healthy persons) whose data served as a reference for EV values in our laboratory and to enable clinical interpretation of levels of platelet derived EV in the hypertensive cohort. Healthy persons were included if they had no known history of clinical disease and no significant risk factors for cardiovascular disease, normal office blood pressure (< 140/90 mmHg), normal routine biochemistry, and an unremarkable physical examination.

The study complied with the Declaration of Helsinki and received approval by the University of Western Australia research ethics committee. All participants provided written consent for the study. Clinical baseline data was collected from the patients including medical history, medication history, serum pathology and blood pressure evaluation.

2.2 | Blood pressure evaluation

Office blood pressure from the brachial artery was measured according to international guidelines. Automated blood pressure was measured after 5-minutes of resting in the sitting position three times with one minute rest periods between measurements. (HEM 907 Automatic Blood Pressure Monitor; Omron Healthcare Co., Kyoto, Japan). Unattended automated office blood pressure (AOBP) was defined as the average of the three measurements.

Ambulatory blood pressure monitoring (ABPM) was performed throughout 24 hours with clinically validated devices (Spacelabs, USA; Mobil-O-Graph IEM GmbH, Germany; OSCAR SunTech, USA). The device was set to measure BP every 15 minutes during day-time (6:00 -22:00 hours) and every 30 minutes during night-time (22:00 to 6:00 hours). 24h-BP, day-BP and night-BP were reported as the average of the successful readings recorded during the period. Participants were instructed to follow their usual daily activities but remain still during measurement. Daily activities were documented in a printed

diary, including bedtimes (adjustment of awake and asleep periods was made if required) and medication intake. Only patients with successful 24hr readings were included in the 24h-BP, day-BP and night-BP analysis (minimum of 70% successful readings including 20 day-time and 7 night-time).[8] Night-time blood pressure dipping was defined as the difference between day-time and night-time mean blood pressure and was expressed as percentage.

2.3 | Assessment of circulating EVs

EVs subpopulations were evaluated by flow cytometry according to the expression of platelet marker (CD41) as described previously by our group.[32] Briefly, venous blood was collected after 10–12 hours of fasting into 3.8% sodium citrate tubes. The first 3 ml of blood was discarded, to avoid platelet activation. Platelet-free plasma (PFP) was obtained by successive centrifugations at 800 g for 10 minutes and double centrifugation at 2500 g for 15 minutes at room temperature (RT). PFP were immediately frozen and stored at -80°C until processing for isolation and quantification. All samples were processed identically and within 1 hr after extraction.

To isolate large EVs, PFP frozen aliquots were thawed at RT and centrifuged at 12000 g for 2 minutes to remove fibrin clots/ aggregates. The supernatant (400 μL) was collected for a subsequent high-speed centrifugation at 20 000 g for 20 minutes. The supernatant was discarded, and the remaining EV-enriched pellet was re-suspended in 300μL ultrafiltered PBS. Re-suspended EVs were incubated for 60 min with fluorochrome-labelled antibodies (CD41-PE-Cy7). The mix was subsequently incubated with Annexin V-FITC at 5% for 10 min and diluted with ultra-filtered annexin binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM CaCl$_2$) before being immediately analyzed on Attune NxT Acoustic Focusing Cytometer. Equivalent concentrations of the respective isotype controls were used to determine the degree of non-specific binding. Acquisition was performed using the lower flow rate (12.5 μL/min min). Forward scatter (FSC), side scatter (SSC), and fluorescence data were obtained with the settings in the logarithmic scale. The concentration of EVs was determined by volumetric cell count in a 50 μL of sample within gate limits established by ApogeeMix (Apogee Flow Systems). The lower detection threshold was set using the 80 nm fluorescent/180 nm silica beads signal. EVs within the established gate limits were identified and quantified based on their binding to Annexin V and reactivity to CD41-PECy7 to define platelet derived EVs (pEVs) (Supplementary material online, Figure S1).

2.4 | Arterial stiffness evaluation

Arterial stiffness was assessed by non-invasive pulse wave analysis (PWA) and pulse wave velocity (PWV) performed with the Sphygmo-Cor XCEL system (AtCor Medical Pty Ltd, Australia) in accordance with manufacturer's recommendations. PWA was performed after a 5-minute rest period in the supine position, an automatic 10 seconds PWA reading was used for data acquisition. Simultaneous measurements through applanation tonometry over the carotid and femoral artery provide the pulse transit time. The time elapsed between carotid and femoral artery sites was used to calculate pulse wave velocity. The capturing time for PWV assessment was set to 10 seconds with a PWV distance and subtraction method. PWV assessments were performed twice, and their average used for further analysis. PWV was expressed as distance/transit time (m/s). Several hemodynamic parameters were documented including central mean arterial pressure (cMAP), aortic augmentation pressure (AP) and augmentation index (AIx). AIx was normalized for a heartbeat of 75 beats per minute to enable comparations and was expressed as percentage.

2.5 | Statistical analysis

For baseline characteristics, continuous variables were expressed as mean $\pm$ standard deviation and categorical variables as frequencies and percentages. Qualitative variables were compared with chi-square test or Fisher's exact test if application conditions were not fulfilled. Comparisons of quantitative variables were evaluated by t-test where appropriate, one-way analysis of variance (ANOVA) for multigroup comparisons. Pearson correlation coefficient was used for correlation analyses for continuous variables, non-parametric tests were applied when necessary.

The association of blood pressure measurements and EVs was assessed by standard regression models with blood pressure measurements as the independent variable and EVs as the dependent variable. A subsequent multivariable regression model was performed, using as covariates demographic or clinical characteristics showing significant differences in univariate analysis, and variables previously reported to have an effect on platelet extracellular vesicles or PWV (eg, age, history of diabetes mellitus, dyslipidemia, hypertension, LDL- levels, glucose levels, and use of antithrombotic and antihypertensive treatment) to test for robustness of the models. Non-linear regression was used when necessary. Log transformation was used in the models to achieve normal distribution of EVs. Statistical significance was considered as a p value < .05. Statistical analysis was conducted using R 4.0.3 software.

3 | RESULTS

3.1 | Baseline characteristics of study participants

A total of 100 participants were included in the study of whom four had to be excluded due to failure to accurately measure EVs (eg, insufficient volume or hemolysis). Thus, samples from 96 participants were included in the present analysis.

The study cohort consisted of 10 healthy, normotensive persons and 86 patients with a diagnosis of hypertension based on office BP measurements at screening. Hypertensive phenotypes were confirmed by ambulatory BP measurements obtained at the initial study visit.[2,8]

Baseline demographics of the study population are summarized in Table 1. The study population had a mean age of 56.1±15.0

TABLE 1 Baseline characteristics of the analyzed patient cohort

	Healthy reference group (No. = 10)	Hypertensive (No. = 86)	Overall (No. = 96)	p-value
Male	5(50.0%)	51(59.3%)	56(58.3%)	.74
Age	33.5 ± 5.76	58.7 ± 13.4	56.1 ± 15.0	<.001
BMI (kg/m^2)	24.2 ± 3.30	30.7 ± 5.84	30.0 ± 5.96	<.001
Diabetes	0(0%)	26(30.2%)	26(27.1%)	.06
Dyslipidemia	0(0%)	62(72.1%)	62(64.6%)	<.001
Coronary artery disease	0(0%)	13(15.1%)	13(13.5%)	.34
White cell count (10*9/L)	5.20 ± 0.933	6.15 ± 1.54	6.07 ± 1.52	.14
Red cell count (10*9/L)	4.80 ± 0.280	4.71 ± 0.513	4.72 ± 0.497	.68
Hematocrit (L/L)	0.420 ± 0.019	0.420 ± 0.037	0.420 ± 0.036	.98
Hemoglobin (g/L)	141 ± 12.7	140 ± 13.3	140 ± 13.2	.89
Platelet count (10*9/L)	280 ± 56.1	243 ± 54.8	246 ± 55.4	.13
Glucose (mmol/L)	5.06 ± 0.477	6.00 ± 1.68	5.94 ± 1.65	.01
HbA1c (%)	5.10 ± 0.100	6.21 ± 1.38	6.15 ± 1.36	<.001
Total cholesterol (mmol/L)	4.60 ± 0.672	4.92 ± 1.17	4.90 ± 1.15	.51
Triglyceride (mmol/L)	0.817 ± 0.299	1.66 ± 1.05	1.60 ± 1.04	<.001
HDL-cholesterol (mmol/L)	1.45 ± 0.383	1.29 ± 0.390	1.30 ± 0.390	.34
LDL-cholesterol (mmol/L)	2.77 ± 0.441	2.89 ± 0.960	2.88 ± 0.932	.76
Creatinine (mmol/L)	84.8 ± 22.8	76.8 ± 27.9	77.3 ± 27.5	.49
eGFR (mL/min/1.73 m^2)	87.2 ± 6.01	84.0 ± 11.6	84.3 ± 11.3	.52
UACR (ug/mg)	0.733 ± 0.554	2.18 ± 4.21	2.02 ± 3.99	.03
Sys AOBP (mmHg)	114 ± 10.6	134 ± 18.1	132 ± 18.4	.001
Dia AOBP (mmHg)	70.6 ± 9.96	79.7 ± 13.6	78.8 ± 13.5	.042
ABPM 24h-SBP (mmHg)	106 ± 5.77	134 ± 13.4	133 ± 14.2	.001
ABPM 24h-DBP (mmHg)	65.0 ± 7.55	77.8 ± 11.3	77.3 ± 11.4	.06
ABPM Day-SBP (mmHg)	108 ± 6.25	136 ± 14.2	135 ± 14.9	.001
ABPM Day-DBP (mmHg)	67.0 ± 8.00	79.8 ± 11.9	79.4 ± 12.0	.06
ABPM Night-SBP (mmHg)	96.3 ± 6.03	123 ± 15.7	122 ± 16.2	.004
ABPM Night-DBP (mmHg)	57.3 ± 5.77	69.3 ± 11.8	68.9 ± 11.8	.09

Data is shown as mean and standard deviation for continuous variables and frequencies and percentages for categorical variables.
AOBP, Automated office blood pressure; ABPM, Ambulatory blood pressure monitoring.

and included 58.3% males. Concomitant diabetes was diagnosed in 27.1% of the study population. The mean office BP across the overall population was 132 ± 18.4/78.8 ± 13.5. Across the 24-h ABPM periods, the mean 24-h, day and night SBP/DBP were 133 ± 14.2/77.3 ± 11.4, 135 ± 14.9/79.4 ± 12, and 122 ± 16.2/68.9 ± 11.8 mmHg, respectively. As expected, 24h ambulatory systolic and diastolic blood pressure was higher in hypertensive patients (n = 86: BP 134±18.1/79.7±13.6 mmHg) compared to healthy participants (n = 10: BP 114.3±10.6/70.6±10 mmHg) (Table 1). Most of the prescribed antihypertensive medication included angiotensin receptor blockers (41.9%), angiotensin converting enzyme inhibitors (39.5%), calcium channel blockers (38.4%) thiazide diuretics (17.4%), potassium-sparing diuretics (4.7%), β-blockers (29.9%), and centrally acting sympatholytic agents (12.7%).

## 3.2 | EV correlation with office and ambulatory blood pressure and pulse wave velocity

The mean level of platelet derived EVs (CD41$^+$/AnnexinV$^+$) were higher in patients with hypertension compared to healthy reference group (10.8±7.9 vs 5.7±3.4 EV/μL; p = .001). Similar results were obtained when comparing only treated hypertensive patients (n = 79) with healthy participants (10.9±7.6 vs 5.7±3.4 EV/μL; p = .001) (Table 2). Night-SBP showed a significant positive correlation with EVs (r = 0.31; p = .003). In contrast, EVs did not show any significant correlation with systolic AOBP (r = 0.12; p = .23), 24hr-BP (r = 0.13; p = .22), or day-BP (r = 0.09; p = .40). Furthermore, EVs were inversely correlated with systolic dipping (r = -0.29; p = .01) (Figure 1A). No significant associations were found for diastolic blood pressure. We performed

TABLE 2 Summary of extracellular vesicles and pulse wave analysis comparisons between healthy participants and hypertensive patients

	Healthy reference group (No. = 10)	Hypertensive (No. = 86)	Overall (No. = 96)	p-value
PWV (m/s)	5.58 ± 1.15	8.44 ± 1.41	8.12 ± 1.65	<.001
cMAP (mmHg)	83.4 ± 10.4	98.0 ± 11.9	96.4 ± 12.5	<.001
AP (mmHg)	4.80 ± 7.41	10.9 ± 6.70	10.2 ± 7.06	.009
AI (%)	1.40 ± 10.5	21.1 ± 11.7	18.8 ± 13.1	<.001
EV Concentration (EV/μL)	5.73 ± 3.37	10.8 ± 7.92	10.3 ± 7.72	.001

Data is shown as mean and standard deviation.
EVs, Extracellular vesicles; PWV, Pulse Wave Velocity; cMAP, Central mean arterial pressure; AP, aortic augmented pressure; AIx, augmentation index.
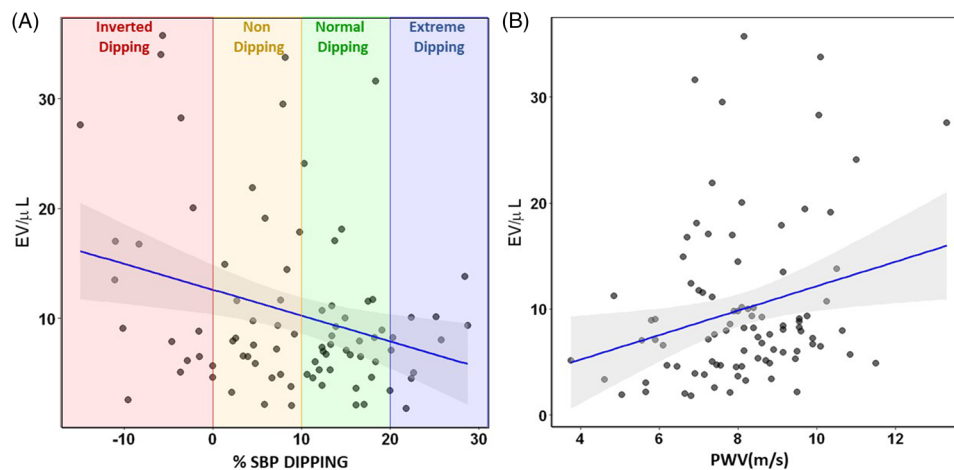
FIGURE 1 Scatterplot of extracellular vesicles, nocturnal dipping, and macrovascular damage. (A) Inverse correlation between EVs and systolic dipping ($p = .001$). (B) Positive correlation between EVs and pulse wave velocity ($p = .02$)

multiple regression analysis for each BP measurement (AOBP, 24h-SBP, day-SBP, and night-SBP) and EVs as dependent variable. Age, presence of diabetes, hypertension, glucose, and LDL- levels as well as the use of antithrombotic and antihypertensive therapy were included as common independent variables to adjust the models (Figure 2). The overall regression was statistically significant (F (9,63) = 2.40, $p = .02$, with an R2 of 0.25). The association between night-SBP and EVs was robust for the adjusted model ($\beta = 0.01$, $p = .001$).

EVs correlated with macrovascular organ damage as assessed by PWV (r = 0.25; $p = .02$) (Figure 1B and Table 3). The overall model including the variables mentioned above was significant (F(9,62) = 2.17, $p = .03$, with an R^2 of 0.24). AP (r = 0.19; $p = .11$) and AIx (r = 0.18; $p = 0.10$) did not show any significance in univariate analysis. The hypertensive group exhibited increased PWV compared to healthy participants (8.44±1.41 vs 5.58±1.15; $p < .001$). Similar results were found for AP (10.9±6.70 vs 4.80±7.41; $p = .01$), AIx (21.1±11.7 vs 1.40±10.5; $p < .001$), and cMAP (98.0±11.9 vs 83.4±10.4; $p < .001$) (Table 2). The results remained significant in the comparison between healthy participants and hypertensive patients on pharmacologic BP treatment. PWV (8.43 ± 1.41 vs 5.58±1.15; $p < .001$), AP (11.3 ± 6.82

vs 4.80±7.41; $p = .007$), AIx (21.5±11.8 vs 1.40±10.5; $p < .001$), and cMAP (97.5 ± 11.8 vs 83.4±10.4; $p < .001$) (Table 3).

All four SBP measurements were strongly correlated with PWV. Interestingly, night-SBP showed the strongest correlation with PWV (r = 0.54; $p < .001$) compared to systolic AOBP (r = 0.50; $p < .001$), day-SBP (r = 0.44; $p < .001$), and 24hr-SBP (r = 0.42; $p < .001$) (Table 3).

3.3 | Association of EVs levels with different phenotypes of hypertension

We performed a sub analysis, to assess possible association of EVs levels with specific phenotypes of hypertension that could be derived from available office BP and ABPM measurements, namely presence of a white coat component or masked HTN.

Hypertensive patients were stratified irrespective of whether or not patients were on antihypertensive therapy in (1) White coat hypertension defined as patients who presented with office BP ≥140/90 mmHg and normal average 24h-BP, day-BP and night-BP; (2) Masked hypertension defined as patients who presented with normal office BP
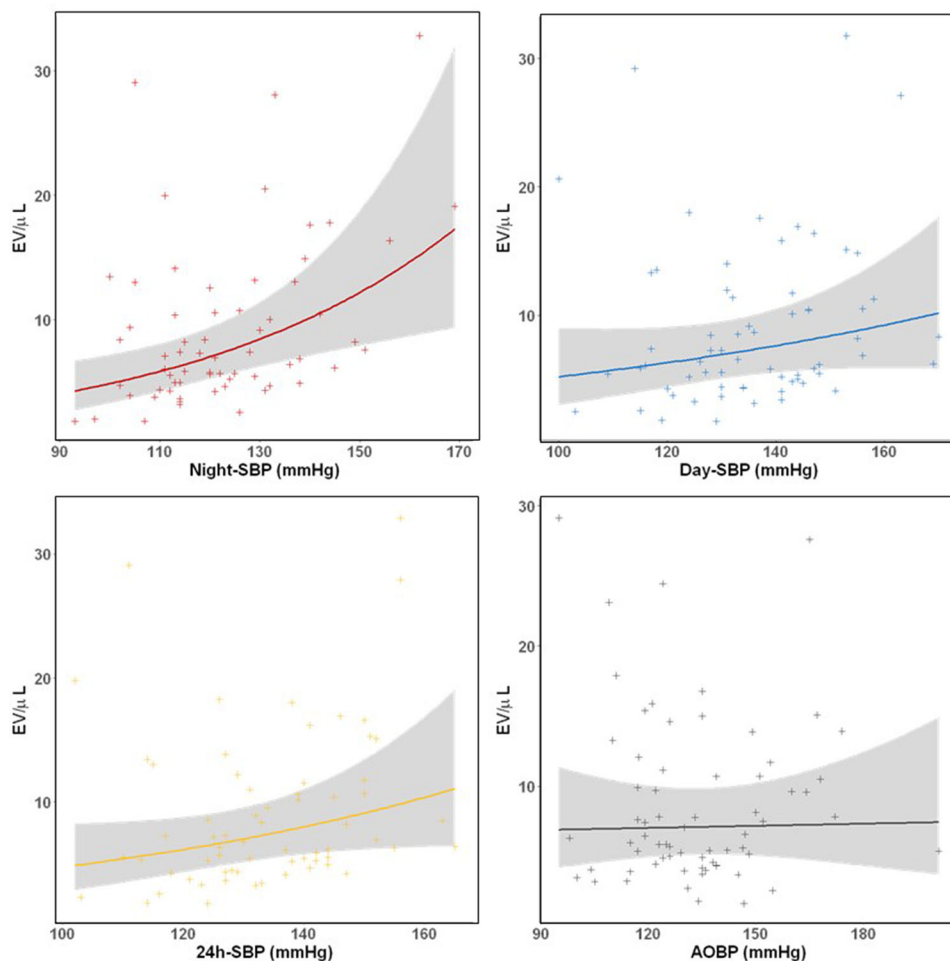
FIGURE 2 Regression analysis of EVs in dependence of systolic blood pressure (Night SBP, Day SBP, 24h SBP, and AOBP). Multiple regression model adjusted for baseline characteristics, including age, presence of diabetes, hypertension, glucose, and LDL- levels, antithrombotic and antihypertensive therapy

(< 140/90 mmHg) and elevated ambulatory BP. As white coat HTN and masked HTN represent two opposite BP patterns we further included in the analysis the two groups that represent similar pattern, healthy participants and sustained high blood pressure (≥140/90 mmHg and elevated 24 average ambulatory BP ≥130/80 mmHg).[2] Patients with controlled hypertension, ie, normal BP for both office and ambulatory BP (n = 22) and patients who couldn't be classified due to incomplete ABPM measurements (n = 2) were not included in this analysis.

Twenty-two patients had controlled office and ambulatory BP, whereas eight patients (8.3%) had white-coat hypertension, 31 (32.3%) had masked HTN, and 23 (24%) had sustained high BP. We compared EV levels among healthy participants and patients presenting with white coat HTN, masked HTN, and sustained high BP the majority of whom were on current antihypertensive treatment. Baseline characteristics are summarized in Table 4. Baseline characteristics were overall well balanced between groups, with the noteworthy exception of age, which was lower in the white coat HTN group. EVs levels showed significant differences between the groups in the one-way ANOVA (p = .002). EVs levels in the group of white coat hypertension (5.84 ± 3.29 EV/uL) were similar to the healthy reference group (5.73 ± 3.37

EV/uL) and significantly lower compared to persons presenting with sustained high blood pressure (13.0 ± 8.47 EV/uL). These comparisons were significant in the post hoc tests for individual group differences (Figure 3). Significant differences between groups were also found for PWV (p < .001), MAP (< .001), and AIx (< .001) (Table 4). PWV and cMAP showed significant differences between the healthy reference group compared with all the other groups.

4 | DISCUSSION

Our main findings can be summarized as follows: (1) hypertensive patients had higher levels of platelet-derived EVs compared to healthy reference participants; (2) circulating EVs were positively correlated with night systolic BP in both univariable and multivariable analysis; (3) EV showed a positive correlation with PWV; (4) night-time SBP demonstrated the strongest correlation with PWV; (5) When comparing different hypertensive phenotypes, we found significant higher levels of EV and PWV in persons presenting with sustained hypertension compared to white coat hypertension and healthy participants.

TABLE 3 Correlation analysis between platelet-derived EV, hemodynamic variables, and different measurements of SBP

	R	t-statistic	p-value
EVs			
Sys-AOBP	0.12	1.21	.23
24h-SBP	0.13	1.23	.22
Day-SBP	0.09	0.84	.40
Night-SBP	0.31	3.01	.003
PWV	0.25	2.46	.02
MAP	0.06	0.58	.57
AP	0.18	1.59	.11
AIx	0.18	1.65	.10
PWV			
Sys -AOBP	0.50	5.47	<.001
24h-SBP	0.42	4.20	<.001
Day-SBP	0.44	4.35	<.001
Night-SBP	0.54	5.62	<.001
cMAP			
Sys -AOBP	0.63	7.79	<.001
24h-SBP	0.43	4.34	<.001
Day-SBP	0.44	4.44	<.001
Night-SBP	0.47	4.81	<.001
AP			
Sys -AOBP	0.37	3.48	<.001
24h-SBP	0.15	1.25	.22
Day-SBP	0.09	0.71	.48
Night-SBP	0.13	1.07	.29
AIx			
Sys -AOBP	0.14	1.29	.20
24h-SBP	0.06	0.49	.63
Day-SBP	0.03	0.24	.81
Night-SBP	0.14	1.23	.22

AOBP, Automated office blood pressure; ABPM, Ambulatory blood pressure monitoring; EVs, Extracellular vesicles; PWV, Pulse Wave Velocity; cMAP, Central mean arterial pressure; AP, aortic augmented pressure; AIx, augmentation index.

Release of EVs from originating cells is a complex process and the contributing factors influencing their release are not completely understood. Whilst much attention has been focused on endothelial derived EVs in the context of hypertension, endothelial dysfunction, and shear stress, the importance of platelet derived EVs in this scenario has been somewhat neglected. Thrombotic events remain one of the most feared and detrimental complications of cardiovascular disease (CVD) and HTN. Platelets have an essential role in the pathophysiology of CVD, as platelet activation is a key aspect to initiate thrombus formation. Numerous clinical factors have been associated with increase platelet activation and worse clinical outcomes (clini-cal presentation, age, diabetes mellitus, chronic kidney disease, smoking status, peripheral artery disease, etc). In the context of HTN the abnormalities in endothelial function, blood flow, and shear conditions are well-recognized factors leading to a prothrombotic state.[5,31,33,34] The endothelial abnormalities due to the constant high-pressure flow is a fundamental mechanism linking HTN and thrombotic complications. Endothelial dysfunction causes an imbalance of the expression of molecules that regulate activation of platelets (tissue factor, plasminogen activator inhibitor-1, etc), decrease in nitric oxide bioavailability, impaired endothelium-dependent vasodilatation, and increased oxidative stress.[5,31,35,36] Shear stress directly affects platelet aggregability as it induces cytoskeletal remodeling of endothelial cells. Furthermore, it has been found that nonactivated platelets can form large aggregates under very high shear due to marked deformation of their membrane[31,33] highlighting the importance of finding advanced biomarkers for platelet activity. It is important to note that platelet EVs represent the most abundant fraction of circulating EVs (~70-90%). Their release can be promoted fundamentally by coagulation, platelet aggregation, or through mechanisms involved in thrombosis (soluble agonists, intracellular calcium release, glycoprotein (GP) IIb/IIIa outside-in signaling). Other factors have been related with pEV release, such as shear stress and chronic inflammation.[13–15,18,37] Importantly, they have a strong procoagulant activity as they display in their surface platelet antigens, selectins, receptors for coagulation factors, anionic phospholipids, and externalized phosphatidylserine.[13,37] Furthermore, EVs can carry proteins, lipids, and miRNAs, hence they can also serve to modulate pathological responses. pEVs can produce thromboxane A2-dependent vasoconstriction,[38] facilitate atherogenesis, enhance expression of cellular adhesion molecules, stimulate inflammation,[18] and platelet and leukocyte.[18,39,40] As thrombotic events including myocardial infarction and stroke are often consequences of hypertension, platelet EVs might represent a suitable biomarker that may help to identify thrombotic risk in early stages.[2,5,31]

Overall, our results are consistent with the literature showing hypertensive patients have higher levels of EVs, PWV, AP, AIx, and cMAP when compared to healthy participants. The results remained consistent when comparing only treated patients with healthy control persons, perhaps reflective of residual macrovascular damage despite treatment. This could be due to time and severity of onset of hypertension, long lasting disease, and individual response to treatment.

Interestingly, only night-time BP showed a statistically significant correlation with circulating EVs levels. This correlation was consistent after adjusting for other risk factors. An association was also evident with the dipping pattern in the univariate analysis, however, the association with EVs was lost in the adjusted multivariable model. This may indicate that EVs release is driven by the absolute night-time BP level regardless of the proportion of BP decrease over night. When comparing different hypertensive phenotypes, we found significantly lower levels of EVs and PWV in the white coat HTN group compared to sustained hypertension, and similar levels of EVs as healthy participants, perhaps supporting previous evidence to suggest that white coat hypertension represents a lower CV risk status than masked HTN or

TABLE 4 Characteristics of hypertensive patients stratified by hypertensive phenotypes

	Healthy reference group (No. = 10)	White coat (No. = 8)	Masked (No. = 31)	Sustained high blood pressure (No. = 23)	p-value
Male	5 (50.0%)	5 (62.5%)	19 (61.3%)	16 (69.6%)	.75
Age	33.5 ± 5.76	49.3 ± 16.6	59.7 ± 13.0	59.6 ± 14.2	<.001
BMI (kg/m^2)	24.2 ± 3.30	31.5 ± 9.00	32.3 ± 5.44	30.4 ± 6.14	.004
Diabetes	0 (0%)	1 (12.5%)	12 (38.7%)	7 (30.4%)	.07
Dyslipidemia	0 (0%)	5 (62.5%)	23 (74.2%)	18 (78.3%)	<.001
Glucose (mmol/L)	5.06 ± 0.477	5.83 ± 1.25	6.44 ± 1.75	5.99 ± 2.13	.42
HbA1c (%)	5.10 ± 0.100	5.70 ± 1.09	6.40 ± 1.26	6.45 ± 2.24	.40
Total cholesterol (mmol/L)	4.60 ± 0.672	4.41 ± 0.875	5.08 ± 1.31	5.25 ± 1.18	.33
Triglyceride (mmol/L)	0.817 ± 0.299	1.21 ± 0.212	1.88 ± 1.13	1.63 ± 0.810	.46
HDL-cholesterol (mmol/L)	1.45 ± 0.383	1.36 ± 0.288	1.23 ± 0.318	1.32 ± 0.457	.57
LDL-cholesterol (mmol/L)	2.77 ± 0.441	2.58 ± 1.04	2.98 ± 1.07	3.19 ± 0.912	.52
Creatinine (mmol/L)	84.8 ± 22.8	73.1 ± 11.4	75.8 ± 17.6	78.6 ± 20.6	.66
eGFR (mL/min/1.73 m^2)	87.2 ± 6.01	86.3 ± 7.50	81.8 ± 12.5	82.6 ± 15.2	.79
Sys AOBP (mmHg)	114 ± 10.6	145 ± 6.22	125 ± 10.6	154 ± 16.2	<.001
Dia AOBP (mmHg)	70.6 ± 9.96	88.5 ± 16.8	74.6 ± 8.87	89.9 ± 14.5	<.001
ABPM 24h-SBP (mmHg)	106 ± 5.77	122 ± 6.46	139 ± 11.0	146 ± 7.17	<.001
ABPM 24h-DBP (mmHg)	65.0 ± 7.55	72.5 ± 7.93	78.9 ± 9.56	85.4 ± 12.6	.02
ABPM Day-SBP (mmHg)	108 ± 6.25	125 ± 8.36	139 ± 12.8	149 ± 7.88	<.001
ABPM Day-DBP (mmHg)	67.0 ± 8.00	74.3 ± 7.19	80.7 ± 10.4	88.0 ± 13.2	.02
ABPM Night-SBP (mmHg)	96.3 ± 6.03	111 ± 10.1	129 ± 13.1	134 ± 14.7	<.001
ABPM Night-DBP (mmHg)	57.3 ± 5.77	66.4 ± 17.8	71.4 ± 9.18	74.9 ± 13.4	.07
Number of antihypertensives	0	1.88 ± 1.55	1.81 ± 1.33	1.70 ± 1.36	.001
PWV (m/s)	5.58 ± 1.15	8.06 ± 1.35	8.38 ± 1.36	9.34 ± 1.54	<.001
cMAP (mmHg)	83.4 ± 10.4	100 ± 20.1	95.2 ± 9.04	109 ± 8.03	<.001
AP (mmHg)	4.80 ± 7.41	6.00 ± 5.79	10.9 ± 6.53	11.5 ± 7.87	.06
AIx (%)	1.40 ± 10.5	14.7 ± 18.7	20.9 ± 12.1	20.5 ± 9.48	<.001
EV (EV/uL)	5.73 ± 3.37	5.84 ± 3.29	9.79 ± 7.65	13.0 ± 8.47	.002

p-values refer to ANOVA between groups. Data is shown as mean and standard deviation for continuous variables and frequencies and percentages for categorical variables. Patients with controlled hypertension (no. = 22) and patients who did not achieve the minimum successful ABPM readings (no. = 2) were not included in this analysis.

AOBP, Automated office blood pressure; ABPM, Ambulatory blood pressure monitoring; EVs, Extracellular vesicles; PWV, Pulse Wave Velocity; cMAP, Central mean arterial pressure; AP, aortic augmented pressure; AIx, augmentation index.

sustained HTN. Conversely, levels of EVs and PWV did not show statistical differences between patients with masked hypertension and sustained HTN.

Arterial stiffness represents a hallmark of early vascular damage and a predictor of cardiovascular outcomes.[41–45] Previous findings suggest that multiple risk factors, their severity, and treatment can affect arterial elasticity. PWV has systematically been used to estimate arterial stiffness and is frequently utilized as a surrogate for cardiovascular events. Our previous findings indicate that elevated nocturnal blood pressure is closely associated with more pronounced organ damage.[46,47] Similarly, we previously demonstrated that reduction of retinal vascularity is associated with an abnormal nocturnal BP dipping pattern.[46] Nocturnal hypertension has also been showed to be an inde-

pendent predictor of PWV.[47] In the analysis of the current population, we found a significant correlation between PWV and EVs.

As mentioned above, in our population EVs showed a significant correlation only with night-SBP. While this finding is in partial contrast to previous investigations reporting a correlation of EVs levels with both systolic and diastolic blood pressure levels,[19–22,48] those studies mainly studied endothelial derived EVs. Additionally, the main differences reported on those studies were especially found in patients with severe hypertension,[22] hypertensive patients with poor BP control,[21] and in hypertensive groups with additional comorbidities (eg, coronary artery disease, hyperlipidemia, and others).[19,49] In a large community-based sample (n = 844), the age- and sex-adjusted models showed a relation of EVs with the presence of hypertension but not with the
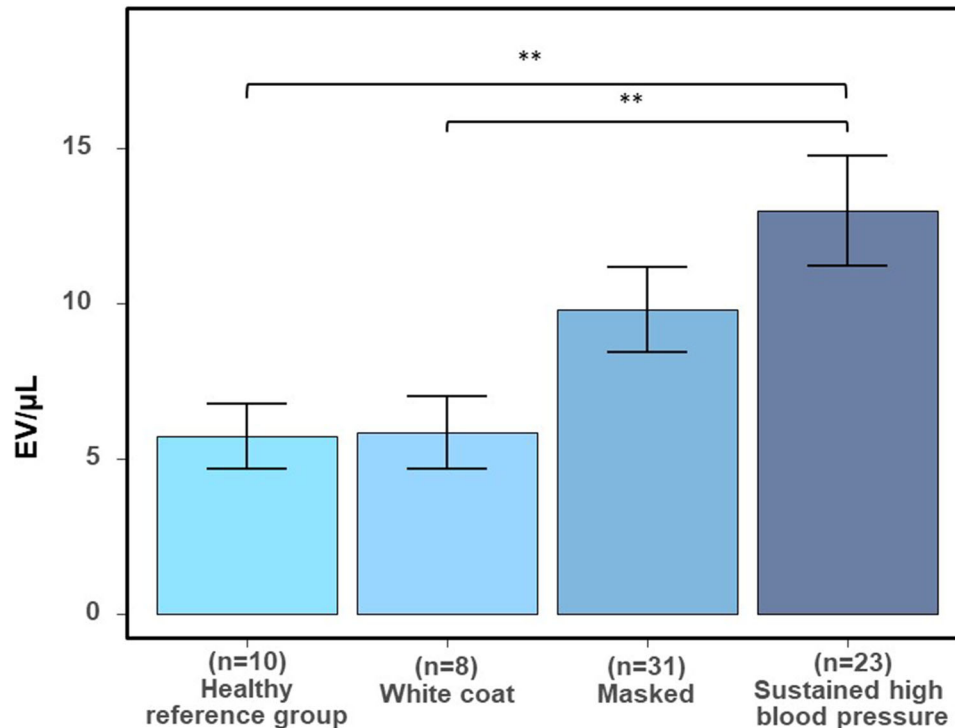
FIGURE 3 Bar graphs of EVs stratified by different hypertensive phenotypes. Values presented are Mean $\pm$ SE. $p = .002$ (ANOVA). Asterisks represent adjusted significant post hoc Tukey test between groups (**$p = .01$)

absolute systolic BP level.[50] The differences between studies might be related to different patient cohorts included, the use of unattended AOBP, the EVs subtype (eg, platelet-derived, endothelial-derived) and surface markers analyzed across different studies. Alternatively, this could reflect a different underlying mechanism responsible of EVs release (eg, acute or chronic damage, vascular injury, and others) as it has been shown that the stimuli involved in their genesis has an important effect on determining the antigens that EVs express.[13] Sasone and associates showed elevated levels of EVs expressing CD62+ and CD144+ during hypertensive emergencies with a decrease in their levels after BP normalization. EVs expressing CD31+/41− do not exhibit such a decrease. Furthermore, CD31+/41− EVs levels during ahypertensive emergency were not statistically different from those in stable hypertensive patients.[20] Recently, a study conducted by Burello and associates characterized EVs in secondary hypertension due to primary aldosteronism. This study demonstrated that patients with primary aldosteronism not only have higher levels of EVs, but also exhibit a different surface profile compared to essential HTN. Furthermore, the marker expression of these patients became similar to patients with essential HTN after adrenalectomy.[48] This suggests that EVs released from the same type of cell (eg, platelet, endothelium, leukocyte), can display different phenotypic markers according to the biological process promoting their release. Finally, a study conducted by Zaldivia and associates showed a reduction of platelet markers and EVs in hypertensive patients successfully treated by renal denervation at 3 and 6 months follow up.[51] Those results imply an additional role of sympathetic nervous system activity in platelet activity and EVs production.

By including different blood pressure assessments, the present analysis providesa more accurate overview of the impact of BP on EVs release, as office BP may be influenced by many factors and ABPM provides better reflection of the BP response to the environment and daily activities, which have significance when evaluating BP phenotypes that are associated with higher cardiovascular risk (masked and nocturnal hypertension, non-dipper pattern). Importantly, the fact that night-BP was correlated with EVs, which have a potent procoagulant activity, is consistent with the physiological coagulation and fibrinolytic activity. Night time confers a pro-coagulable state due to the peak of plasminogen activator inhibitor-1 and the nadir of tissue-type plasminogen activator.[5] Additionally, PWV showed a better correlation with night-BP. These two key findings have special clinical relevance as it aligns with evidence from large studies pointing out the superiority of nocturnal BP in predicting cardiovascular events.[5,10–12] The Dublin Outcome Study including 5292 patients demonstrated night-BP as the strongest predictor for mortality over a median follow-up of 8.4 years, with a hazard ratio of 1.21 (1.15 to 1.27; $p < .001$) for night-time BP and 1.12 (1.06 to 1.18; $p < .001$) for day-time BP.[10] Results from the Pressioni Arteriose Monitorate e Loro Associazioni (PAMELA) study (n = 2051) showed similar results, night-BP was associated with a higher risk of cardiovascular death.[11]

Our study has some limitations. First, given the sample size and the cross-sectional nature of this analysis, a causal relationship cannot necessarily be inferred and can only be interpreted as a hypothesis generating. Of note, the overall sample size of our cohort compares favorably to several other studies exploring the role of EV with

sample sizes ranging from 41 to 86 persons,[19,22,48] Second, a small proportion of our participants had some missing pathology data or were unable to achieve the required minimum successful number of readings during the ABPM. Nevertheless, our results remained consistent when these factors were taken into account. Finally, we include treated and untreated hypertensive patients, and even though we included the pharmacological treatment in the model, further longitudinal studies are required to confirm these results and determine whether different pharmacological interventions may have an effect on EVs release and macrovascular damage.

In conclusion, our results showed that EVs release is correlated with night blood pressure, which has been well-recognized as a predictor for cardiovascular events. In contrast, EV levels were lower in the white coat HTN phenotype, which in general is characterized by less pronounced or no organ damage compared to sustained hypertension. PWV as a surrogate of macrovascular damage was also associated with EV release. The robust association of EVs with night-time BP, together with the fact that the former was a better predictor of macrovascular damage, highlights the importance of characterizing the different hypertensive phenotypes and the possible use of EVs to evaluate vascular damage. Taken together, our findings suggest that EVs might represent a potential early biomarker that reflects the underlying vascular health status and could potentially be utilized as an integrative biomarker of overall vascular health. Larger dedicated studies are needed to understand the pathological mechanisms, causal structure, and bioactive properties of EVs, as well as the potential utility of EVs assessment as a relevant biomarker of overall vascular status in real-world clinical practice.

CONFLICT OF INTEREST

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AUTHOR CONTRIBUTIONS

LMLG contributed to the conceptualization, carried out the implementation of clinical work, performed sample and data collection, planned and carried out the standardization methods for flow cytometry, performed experiments, performed data analysis and wrote the manuscript with input from all authors. **DB, EB, VBM** conceived and designed the standardization methods for flow cytometry. **JC, SR, JMN, RC** contributed to sample and data collection. **MPS**. conceived the study and supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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