

BMJ Open Extracellular vesicle protein levels are related to brain atrophy and cerebral white matter lesions in patients with manifest vascular disease: the SMART-MR study

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ABSTRACT

Objectives: Extracellular vesicles (EVs) and their protein levels have been identified as a potential risk marker for the development of vascular disease. In the present study, we assessed whether levels of four previously identified EV proteins (cystatin C, serpin G1, serpin F2 and CD14) are associated with cerebral white matter lesions (WMLs) and brain atrophy.

Design: Cohort study; cross-sectional and prospective.

Setting: Single centre, secondary and tertiary setting.

Participants: 1309 patients with manifest vascular disease from the Second Manifestations of ARterial disease-MR (SMART-MR) study, of which 994 had successful brain MRI and EV protein level measurements.

Outcomes: WML and brain parenchymal fraction (BPF), as parameter for brain atrophy, at baseline and follow-up.

Statistical methods: The relationship between EV protein levels and WML volume (expressed as log transformed percentage of intracranial volume) and BPF (expressed percentage of intracranial volume) on 1.5 T brain MRI was assessed with multivariable linear regression modelling. Subsequently, the relationship between baseline EV protein levels and progression of atrophy and WML was analysed in 534 patients, in whom a follow-up MRI was obtained after 4 years.

Results: Higher EV-cystatin C and EV-CD14 were significantly associated with larger WML volume (linear regression coefficient (95% CI) 0.10 log %/SD (0.04 to 0.17) and 0.14 log %/SD (0.07 to 0.20), respectively. Higher EV-CD14 was associated with more brain atrophy (-0.14%/SD; -0.27 to -0.01). Baseline EV-CD14 was significantly associated with increase of WMLs (0.11 log %/SD (0.04 to 0.18)). No relationship with EV-serpins was observed at baseline or at follow-up.

Conclusions: EV proteins cystatin C and CD14 are related to cerebral WMLs and the progression of brain atrophy in patients with manifest vascular disease, potentially identifying EVs in the aetiology of structural brain changes.

Strengths and limitations of this study

- Relatively large sample size.
- Follow-up measurements of brain parameters.
- Only four of the numerous protein levels were measured.
- Unclear how extracellular vesicles interact with the blood-brain barrier.

INTRODUCTION

Patients with manifest vascular disease often have morphological changes in the small vessels of the brain, characterised by white matter lesions (WML) on MRI.¹ The exact underlying pathophysiological mechanism regarding WML remains unclear. WML have been associated with cognitive decline² and may explain the increased risk for cognitive decline in patients with manifest vascular disease.³⁻⁴ Cognitive decline and vascular disease have more in common as they share important risk factors such as obesity, hypertension, dyslipidaemia and diabetes.^{5,6}

Recently, extracellular vesicles (EVs) have been identified as a novel, independent risk marker for the occurrence of vascular disease.^{7,8} EVs are membrane-shed vesicles, between 50 and 1000 nm in diameter, released in the extracellular space after cell activation or apoptosis, and include various phenotypes such as microparticles and exosomes.^{9,10} EVs are defined by size and antigen expression, which depend on their originating cell type.^{11,12} Release of EVs allows cells to influence (patho)physiological processes over distance in contrast to cell-cell contact.

EVs transfer proteins, mRNA, miRNA and bioactive lipids from one cell to another cell

by either fusion or internalisation with target cells.¹³ For example, monocyte-derived EVs are internalised by endothelial cells and activate the nuclear factor- κ B (NF κ B) pathway and expression of adhesion molecules on these endothelial cells, amplifying inflammation.¹⁴ EVs are procoagulant, as they carry phosphatidylserine on their membrane, which facilitates the assembly of components of the clotting cascade.¹⁵ In addition, EVs also carry the procoagulant protein tissue factor, which is primary cellular activator of the clotting cascade.¹⁶ It has been demonstrated that atherosclerotic plaques had 200-fold higher levels of EVs in comparison with plasma of the same study participants,¹⁷ suggesting an active role of EVs in atherothrombotic disease rather than a trigger of disease onset.

Virtually every cell is capable of producing EVs, including brain cells such as neurones and astrocytes.¹⁸ Little is known about the role EVs play in the development of brain atrophy or small vessel disease. However, microparticles, a subclass of EVs, have been shown to influence arterial stiffness¹⁹ which is associated with cerebral small vessel disease.^{20–22} Alternatively, platelet-derived microparticles have been suggested to play a role in the formation of cerebral microthrombi,²³ a known initiator of brain atrophy and subsequent cognitive decline. Exosomes, another subclass of EVs, have been shown to pass the blood–brain barrier (BBB).^{24 25} In a murine model, a specific gene knockdown in cerebro was achieved through the actions of peripherally infused modified exosomes containing specific RNA.²⁴

We hypothesised that EV protein levels of cystatin C, serpin G1, serpin F2 and CD14 in peripherally circulating plasma EVs, which have been related to the occurrence of vascular disease previously, also are associated with the presence or increase of brain atrophy or with small vessel disease. Therefore, we investigated the relationship between levels of these four EV proteins extracted from plasma, brain parenchymal fraction (BPF) and WMLs in a cohort of patients with clinically manifest vascular disease.

METHODS

SMART-MR study

The study population consisted of patients participating in the Second Manifestations of ARterial disease-MR (SMART-MR) study, a prospective cohort study aimed at investigating brain changes on MRI in 1309 patients with clinically manifest vascular disease. Details of the design and participants have been described elsewhere.^{1 26} In brief, between May 2001 and December 2005, all patients newly referred to the University Medical Center Utrecht, The Netherlands with manifest coronary artery disease (CAD), cerebrovascular disease (CVD), peripheral arterial disease (PAD) or an abdominal aortic aneurysm (AAA), and without MR contraindications, were invited to participate. CVD was defined as a recent diagnosis of ischaemic stroke, transient ischaemic attack

or amaurosis fugax. CAD was defined as a recent diagnosis of angina pectoris, myocardial infarction or coronary revascularisation (coronary artery bypass graft or coronary angioplasty). PAD consisted of those with a clinical diagnosis of Fontaine stage 2, 3 or 4. AAA was defined as an AAA of ≥ 3.0 cm or recent aneurysm surgery.

Excluded were patients with a terminal disease, active malignancy, those not independent in daily activities and those referred back to the referring specialist immediately after one visit. After inclusion, the patients underwent a standardised vascular screening including assessment of vascular risk factors and non-invasive measurement of subclinical atherosclerosis in addition to MRI of the brain. Risk factors, medical history and functioning were assessed with questionnaires that the patients completed prior to their visit. Of the original 1309 patients comprising this cohort, 1232 successfully underwent MRI measurement. For 994 of these patients, material for EV protein level measurement could be retrieved and had at least one EV protein level measurement. Between January 2006 and May 2009, all the participants still alive (n=1238) were invited for follow-up measurements, including a second MRI of the brain. For 534 patients, data on follow-up MRI as well as baseline EV protein levels were available; and written informed consent was obtained from all the participants.

MRI protocol

MR investigations were performed on a 1.5 T whole-body system (Gyrosan ACS-NT, Philips Medical Systems, Best, The Netherlands). The protocol consisted of a transversal T1-weighted gradient-echo sequence (repetition time (TR)/echo time (TE): 235/2 ms; flip angle, 80°), a transversal T2-weighted turbo spin-echo sequence (TR/TE: 2200/11 and 2200/100 ms; turbo factor 12), a transversal T2-weighted fluid attenuating inverse recovery (FLAIR) sequence (TR/TE/inversion time (TI): 6000/100/2000 ms) and a transversal inversion recovery (IR) sequence (TR/TE/TI: 2900/22/410 ms) (field of view mm; matrix size; slice thickness, 4 mm; no gap; 38 slices).

Brain segmentation

The T1-weighted gradient echo, IR sequence and FLAIR sequence were used for brain segmentation with the probabilistic segmentation technique that has been described elsewhere.^{27 28} This segmentation programme distinguishes cortical grey matter, normal appearing white matter, sulcal and ventricular cerebrospinal fluid (CSF) and lesions. The results of the segmentation analysis were visually checked for the presence of infarctions and adapted if necessary to make a distinction between WMLs and infarction volumes. Total brain volume was calculated by summing the volumes of grey and normal appearing white matter and, if present, the volumes of WML and infarcts. All volumes cranial to the foramen magnum were included. As a result, the total brain volume includes both hemispheres, brainstem and cerebellum. Total intracranial volume (ICV) was calculated

by summing the total brain volume and the volumes of the sulcal and ventricular CSF.

White matter lesions

Periventricular WMLs were defined as WML adjacent to or within 1 cm of the lateral ventricles. Deep WMLs were defined as lesions located in deep white matter tracts or without adjoined periventricular lesions. If white matter hyperintensities on T2-weighted images were also hypointense on T1-weighted and FLAIR images, they were considered infarcts and therefore distinguished from WML.

Brain parameters

Two brain parameters were used for analyses: WML, which was the sum of periventricular and deep WML in millilitres, expressed as percentage of ICV and BPF, which was determined by calculating the total brain volume in millilitres as percentage of ICV. By including the percentages of ICV instead of crude brain parameters, correction for differences in head size was applied.

Extracellular vesicle measurements

The complete measurement methods are described elsewhere.⁷ EVs were isolated using ExoQuick (SBI) according to the manufacturer's protocol. Briefly, 150 μ L EDTA plasma was centrifuged for 15 min at 3000g. The supernatant was filtered over a 0.45 μ m Spin-X filter (Corning), which was flushed with preheated phosphate buffered saline (37°C) and 38 μ L ExoQuick solution was added to the filtrate. After vortexing, the sample was stored overnight at 4°C. The following day, the sample was centrifuged at 1500g for 30 min at room temperature. After removing the supernatant, the pellet was lysed in 100 μ L Roche Complete Lysis-M with protease inhibitors (EDTA free). Subsequently, the sample was filtered over a 0.22 μ m Spin-X filter (Corning) and protein level was determined using a Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA) before storing the sample at -80°C. After thawing, the lysed sample was diluted 20 \times with Roche complete lysis-M buffer. Of this diluted sample, 50 μ L was analysed in a multiplex immunoassay on levels of cystatin C, serpin G1, serpin F2 and CD14, using a Bio-Rad Bioplex 200 system as described elsewhere.²⁹ Capture antibody, biotinylated detection antibody and antigen of all four proteins were purchased from research and development systems.

Data analyses

Central estimators and their variance measures were calculated for baseline characteristics. Multivariable linear regression was performed to assess the cross-sectional relationship between EV protein level in pg/mL and WML in per cent or BPF in per cent. The fully adjusted model included age, gender, systolic blood pressure, smoking and type 2 diabetes, as these covariates are

considered to be confounding factors in the suggested relationship between EV protein levels and measured structural brain changes.

To fulfil linear regression assumptions, WML volumes were naturally log-transformed as they showed skewed distributions. In order to directly compare the strengths of associations, linear regressions were performed per SD increase in EV protein level. Second, we assessed the prospective relationship between baseline EV protein levels and progression in brain parameters on MRI. This was carried out by relating baseline EV protein levels to follow-up WML or BPF. Besides adjustment for age, gender, systolic blood pressure, smoking and type 2 diabetes, these analyses were additionally adjusted for baseline WML or BPF and follow-up time. The results are expressed as linear regression coefficients and 95% CIs.

RESULTS

Baseline characteristics

The patient's characteristics are displayed in table 1. Seventy-nine per cent of the initial patients were men and the mean age was 59 \pm 10 years. The majority of the patients had a (recent) medical history of CAD (58%), followed by CVD (26%), PAD (24%) and AAA (10%). After a mean interval time of 3.9 \pm 0.4 years, 534 patients underwent a second MRI of the brain. This follow-up cohort comprised 80% men and a mean baseline age of 58 \pm 9 years, which corresponded with a mean follow-up age of 62 \pm 9 years. For the baseline comparisons of the two groups, refer to online supplemental table S1.

Cross-sectional association between EV protein levels and WML or BPF

In the fully adjusted model, higher EV-cystatin C and EV-CD14 were associated with more volume of WML. Regression coefficients per SD EV-protein were 0.10 (95% CI 0.04 to 0.17) for EV-cystatin C and 0.14 (95% CI 0.07 to 0.20) for EV-CD14 (table 2). Also, higher EV-CD14 was associated with a statistically significantly lower BPF (regression coefficient per SD EV-CD14 -0.14 (95% CI -0.27 to -0.01); table 3). SD increases of EV-serpin G1 and EV-serpin F2 were not related with WML or BPF at baseline.

In general, adjustment for age and gender in model 2 altered the effect estimates substantially, whereas the impact that systolic blood pressure, smoking and type 2 diabetes had on the occurrence relation was relatively small. Tests for interaction by age and gender yielded non-significant results (data not shown).

Prospective associations between EV protein levels and WML or BPF

In the fully adjusted model, higher EV-CD14 was associated with increase of WML volume (regression coefficient=0.11 (95% CI 0.04 to 0.18)) as is displayed in table 2. EV-cystatin C, EV-serpin G1 and EV-serpin F2 were not related with increase of WML or BPF.

Table 1 Baseline patient characteristics

	Baseline cohort N=994	Follow-up patients N=534	Non-FU patients N=460
Age (years)*	59±10	58±9	60±11
Male gender, n (%)	784 (79)	426 (80)	358 (78)
Body mass index (kg/m ²)*	26.9±3.8	26.9±3.6	26.8±4.1
Waist circumference (cm)*	95±11	95±11	96±12
Blood pressure (mm Hg)*			
Systolic	144±22	142±20	145±23
Diastolic	83±12	82±11	83±12
LDL cholesterol (mmol/L)*	2.8±0.93	2.8±0.90	2.9±0.97
HDL cholesterol (mmol/L)*	1.30±0.38	1.31±0.38	1.28±0.37
Triglycerides (mmol/L)†	1.47 (1.09–2.07)	1.41 (1.05–2.07)	1.47 (1.09–2.07)
Glucose (mmol/L)*	6.3±1.9	6.2±1.8	6.4±1.9
HsCRP (mmol/L)†	1.8 (0.9–3.8)	1.6 (0.8–3.5)	1.8 (0.9–3.8)
eGFR (mL/min/1.73 m ²)*	77.7±17.9	79.7±17.1	75.4±18.6
Homocysteine (µmol/L)*	14.0±6.0	13.5±4.9	14.6±7.0
Prevalent type 2 diabetes, n (%)	156 (16)	71 (13)	85 (19)
Metabolic syndrome, n (%)‡	369 (37)	184 (35)	185 (40)
Smoking, n (%)			
Never	170 (17)	100 (18)	70 (15)
Ever	454 (46)	257 (48)	197 (43)
Current	370 (37)	177 (33)	193 (42)
Pack years smoking*	23.0±20.5	23.0±20.1	23.1±21.0
History of vascular disease, n (%)			
Cerebrovascular disease	261 (26)	144 (27)	117 (25)
Coronary artery disease	580 (58)	325 (61)	255 (55)
Peripheral artery disease	241 (24)	108 (20)	133 (29)
Aneurysm of the abdominal aorta	99(10)	37 (7)	62 (14)
Medication, n (%)			
Platelet-aggregation inhibitors	740 (74)	407 (76)	333 (72)
Blood pressure-lowering agents	702 (71)	382 (72)	320 (70)
Lipid-lowering agents	682 (69)	388 (73)	294 (64)
Oral anticoagulants	80 (8)	37 (7)	43 (9)
White matter lesions (log%)*	−2.1 (1.1)	−2.3 (1.1)	−1.9 (1.1)
Brain parenchymal fraction (%)*	79 (3)	79 (3)	79 (3)

*Mean±SD.

†Median (IQR).

‡Defined according to the National Cholesterol Education Programme ATPIII-revised guidelines.

eGFR, epidermal growth factor receptor; HDL, high-density lipoprotein; HsCRP, high-sensitivity C reactive protein; LDL, low-density lipoprotein.

DISCUSSION

In patients with various clinical manifestations of vascular disease, EV protein levels of cystatin C and CD14 in plasma are associated with larger WML and more brain atrophy. Furthermore, higher EV-CD14 levels are associated with an increase in WML during 4 years of follow-up. This relation is independent of age, gender, systolic blood pressure, smoking and type 2 diabetes.

The role of cystatin C, a cysteine protease inhibitor, in the process of degenerative disorders in the brain is unclear. In vitro and murine studies have shown that cystatin C binds to amyloid-β (aβ), resulting in the inhibition of aβ deposition,^{30–32} the characteristic component of the neuritic plaques that identify patients with Alzheimer's disease. This protective role of cystatin C was supported in a cohort study³³ where lower levels of serum cystatin C were associated with higher risk for future Alzheimer's disease in men. However, higher

levels of serum cystatin C have also been associated with cognitive decline^{34–35} and with WML³⁶ in large population-based cohort studies. In WML, the cystatin C expression is upregulated in astrocytes, presumably due to a self-defence response in the process of white matter degeneration³⁷. Our results, based on EV-cystatin C, show similar results as increasing EV-cystatin C is related to increased volume of WML. Cystatin C is secreted by all human tissues³⁸ which presumably concomitantly shed cystatin C⁺ EVs in order to directly influence more distant processes.

CD14 is a cofactor for Toll-like receptor 4, which regulates NFκB expression, indicating a role for CD14 in inflammation.³⁹ CD14 is expressed on monocytes⁴⁰ and it is therefore assumable that CD14⁺ EVs originate from these monocytes. In our study, EV-CD14 levels were related to the volume and increase of WML. This indicates an EV-initiated inflammatory response in dealing

Table 2 EV protein levels and WMLs

	Model	WML	
		WML at baseline n=994	WML at follow-up* n=534
EV-cystatin C (pg/ μ g)	I	0.27 (0.20–0.34)	0.06 (–0.02–0.13)
Mean 10.6	II	0.10 (0.04–0.17)	0.01 (–0.06–0.08)
SD 5.3	III	0.10 (0.04–0.17)	0.01 (–0.06–0.08)
EV-serpin G1 (pg/ μ g)	I	–0.03 (–0.10–0.05)	0.02 (–0.05–0.08)
Mean 142.2	II	–0.02 (–0.08–0.04)	0.02 (–0.04–0.08)
SD 85.6	III	–0.03 (–0.08–0.05)	0.03 (–0.03–0.09)
EV-serpin F2 (pg/ μ g)	I	0.01 (–0.066–0.08)	–0.01 (–0.08–0.05)
Mean 43.3	II	0.03 (–0.034–0.09)	0.01 (–0.06–0.07)
SD 30.4	III	0.03 (–0.035–0.09)	0.01 (–0.05–0.07)
EV-CD 14 (pg/ μ g)	I	0.23 (0.16–0.30)	0.12 (0.04–0.18)
Mean 12.2	II	0.14 (0.08–0.20)	0.10 (0.03–0.17)
SD 3.9	III	0.14 (0.07–0.20)	0.11 (0.04–0.18)

Bold typeface indicates statistically significant results.

Regression coefficients: increase in WML (log%) per 1SD increase in EV protein level (pg/mL).

Model I: univariable model.

Model II: adjustment for age and gender.

Model III: Model II with additional adjustment for systolic blood pressure, pack years smoking and prevalent type 2 diabetes mellitus.

*Models include additional adjustment for baseline WML and follow-up time.

EV, extracellular vesicle; WMLs, white matter lesions

with WML. These findings do not stand alone as several inflammatory biomarkers such as lipoprotein-associated phospholipase A2 and myeloperoxidase (MPO) have been associated with WML.⁴¹ Although the effects of EV-CD14 on WML and BPF have not been assessed previously, soluble CD14 has been associated with cognitive impairment and brain atrophy in patients with HIV.⁴² Likewise, higher plasma levels of EV-CD14 were inversely related to BPF at baseline in our study.

EV-Serpin G1 (C1-inhibitor) and EV-Serpin F2 (α 2-antiplasmin) were not associated with WML or BPF in our study, suggesting no substantial role of (inhibited)

fibrinolysis in the pathophysiology surrounding WML or brain atrophy. To our understanding, this is the first report on these serpins and their potential role in the aetiology of WML and brain atrophy.

Whether and how EVs or EV protein levels might directly influence ischaemic WMLs or brain volume is not known. Several mechanisms are proposed. We assume that EVs have BBB penetrating properties, based on recent data regarding exosomes.^{24 25} It could also be that EVs interact with endothelial cells through receptor-mediated endocytosis. Once fused with these endothelial cells, EV protein levels might activate inflammation

Table 3 Extracellular vesicle protein levels and BPF

	Model	BPF	
		BPF at baseline n=994	BPF at follow-up* n=534
EV-cystatin C (pg/ μ g)	I	–0.69 (–0.86–0.51)	–0.15 (–0.26–0.04)
Mean 10.6	II	–0.12 (–0.26–0.02)	–0.06 (–0.16–0.04)
SD 5.3	III	–0.11 (–0.24–0.03)	–0.05 (–0.16–0.05)
EV-serpin G1 (pg/ μ g)	I	–0.00 (–0.18–0.17)	0.04 (–0.05–0.14)
Mean 142.2	II	0.01 (–0.12–0.14)	0.02 (–0.06–0.11)
SD 85.6	III	0.10 (–0.12–0.14)	0.03 (–0.06–0.11)
EV-serpin F2 (pg/ μ g)	I	0.05 (–0.12–0.23)	0.06 (–0.04–0.16)
Mean 43.3	II	–0.10 (–0.23–0.03)	–0.01 (–0.10–0.08)
SD 30.4	III	–0.06 (–0.19–0.07)	0.01 (–0.09–0.10)
EV-CD 14 (pg/ μ g)	I	–0.45 (–0.62–0.28)	–0.06 (–0.17–0.05)
Mean 12.2	II	–0.19 (–0.32–0.06)	–0.05 (–0.15–0.05)
SD 3.9	III	–0.14 (–0.27–0.01)	–0.03 (–0.13–0.07)

Bold typeface indicates statistically significant results.

Regression coefficients: increase in BPF (%) per 1SD increase in EV protein level (pg/mL).

Model I: univariable model.

Model II: adjustment for age and gender.

Model III: model II with additional adjustment for systolic blood pressure, pack years smoking and prevalent type 2 diabetes mellitus.

*Models include additional adjustment for baseline WML and follow-up time.

BPF, brain parenchymal fraction; EV, extracellular vesicle; WML, white matter lesion.

cascade signalling, leading to vascular damage and subsequent arterial stiffness. It might also activate haemostasis cascade signalling leading to the formation of (micro) thrombi. Alternatively, through endocytosis by endothelial cells, EV protein levels might influence the structural integrity of the endothelial layer, resulting in a greater BBB permeability. Neurodegenerative disorders such as Alzheimer's disease⁴³ and multiple sclerosis⁴⁴ have been associated with BBB integrity loss. The mechanism behind this loss is unclear and perhaps EV vesicles play a role within this process. Future work will have to test these hypotheses. If true causation between EV protein levels and brain changes would be the case, strategies on influencing EV protein levels should be developed.

The strengths of the present prospective cohort study include the relatively large sample size with various EV protein measurements and MRIs of the brain at baseline and 3.9 years follow-up, which makes reversed causality less likely. Another strength of the study is the use of automated segmentation techniques to obtain brain parameters, which provides more precise and objective estimates than visual rating scales⁴⁵ and enables accurate measurement of small volume changes over time.⁴⁶

Potential study limitations should also be considered. First, data of only 54% of the surviving cohort was used to measure change in WML or BPF. The patients who were physically able to undergo the follow-up MRI were most likely healthier than those who did not participate, which might have led to the dilution of associations. Second, only four of the numerous proteins on the surface or within EVs were measured. It could be that besides these vascular risk markers, various other EV proteins have their aetiology in the structural brain changes. Third, we assume that plasma EVs which hold the proteins measured in the present study have the ability to cross the BBB in order to have their effects on the brain, influencing WML and BPF. Unfortunately, no data are available whether these EVs are indeed present within the brain, and it would be interesting to know whether these EV proteins are present in CSF and have a non-cerebral origin. If these EVs can truly pass the BBB and influence the pathophysiological processes in the brain, it automatically gives rise to potential therapeutical purposes analogous to exosomes, as these vesicles can deliver complex drugs directly into the brain, a key step in the treatment of brain diseases or brain changes.⁴⁷

In conclusion, EV-cystatin C and EV-CD14 levels are related with the volume of WML and with BPF on the brain MRI. EV-CD14 levels are related with an increase in the total volume of WML during follow-up. EV proteins levels may be causally related to brain changes in patients with clinically manifest vascular disease.

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