# Microvesicle 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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# Microvesicle 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*

Microvesicles (MVs) 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 4 previously identified MV 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 center, secondary and tertiary setting

*Participants*

1309 Patients with manifest vascular disease from the Second Manifestations of ARTerial disease-Magnetic Resonance (SMART-MR) study, of which 994 had successful brain Magnetic Resonance Imaging (MRI) and MV protein level measurements

*Outcomes*

WMLs and brain parenchymal fraction (BPF), as parameter for brain atrophy, at baseline and follow-up

*Statistical methods*

The relation between MV protein levels and WML volume (expressed as log transformed percentage of intracranial volume) and BPF (expressed percentage of intracranial volume) on 1.5 Tesla brain MRI was assessed with multivariable linear regression modeling. Subsequently, the relation between baseline MV protein levels and progression of atrophy and WMLs was analyzed in 534 patients, in whom a follow-up MRI was obtained after 4 years.

*Results*

MV-Cystatin C and MV-CD14 were significantly associated with larger WML volume (linear regression coefficient (95% confidence interval) 0.10 log %/SD (0.04 to 0.17) and 0.14 log %/SD (0.07 to 0.20), respectively. Higher MV-CD14 was associated with more brain atrophy (-0.14 % /SD; -0.27 to -0.01). Baseline MV-CD14 was significantly associated with increase of white matter lesions (0.11 log %/SD (0.04–0.18). No relations with MV-Serpins were observed at baseline or at follow-up.

*Conclusions*

Microvesicle proteins Cystatin C and CD14 are related to cerebral white matter lesions and the progression of brain atrophy in patients with manifest vascular disease, potentially identifying MVs in the etiology of structural brain changes.

## Introduction

Patients with manifest vascular disease often have morphologic changes in the small vessels of the brain, characterized by white matter lesions (WML) on magnetic resonance imaging (MRI) [1]. The exact underlying pathophysiological mechanism regarding WMLs remains unclear. WMLs have been associated with cognitive decline [2] 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, dyslipidemia and diabetes [5, 6].

Recently, microvesicles (MVs) have been identified as a novel, independent risk marker for the occurrence of vascular disease [7, 8]. MVs are membrane shed vesicles, between 50 and1000 nm in diameter, released in the extracellular space after cell activation or apoptosis, and include various phenotypes such as microparticles and exosomes [9,10]. MVs are defined by size and antigen expression, which depends on their originating cell type [11,12]. Release of MVs allows cells to influence (patho)physiological processes over distance in contrast to cell-cell contact.

MVs transfer proteins, mRNA, miRNA and bioactive lipids from one cell to another cell by either fusion or internalization with target cells [13]. For example, monocyte-derived MVs are internalized by endothelial cells and activate the nuclear factor-κB pathway and expression of adhesion molecules on these endothelial cells, amplifying inflammation [14]. MVs are procoagulant, as they carry phosphatidylserine on their membrane, which facilitates the assembly of components of the clotting cascade [15]. In addition, MVs 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 MVs in comparison with plasma of the same study subjects [17], suggesting an active role of MVs in atherothrombotic disease rather than a trigger of disease onset.

Virtually every cell is capable of producing MVs, including brain cells such as neurons and astrocytes [18]. Little is known about the role MVs play in the development of brain atrophy or small vessel disease. However, microparticles, a subclass of MVs, have been shown to influence arterial stiffness [19], 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 MVs, 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 hypothesized that MV protein levels of Cystatin C, Serpin G1, Serpin F2 and CD14, in peripherally circulating plasma MVs, 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 relation between levels of these 4 MV proteins extracted from plasma, and brain parenchymal fraction (BPF) and white matter lesions (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-Magnetic Resonance (SMART-MR) study, a prospective cohort study aimed at investigating brain changes on magnetic resonance imaging (MRI) in 1,309 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 ischemic stroke, transient ischemic attack or amaurosis fugax. CAD was defined as a recent diagnosis of angina pectoris, myocardial infarction or coronary revascularization (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 abdominal aortic aneurysm of ≥3.0 centimeter 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 1 visit. After inclusion, patients underwent a standardized 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 MV protein level measurement could be retrieved and had at least one

MV protein level measurement. Between January 2006 and May 2009, all participants still alive (n = 1,238) 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 MV protein levels were available. The SMART-MR study was approved by the ethics committee of the University Medical Center Utrecht, and written informed consent was obtained from all participants.

*Magnetic resonance imaging protocol*

MR investigations were performed on a 1.5-Tesla whole-body system (Gyroscan 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 ms 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, 4.0 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 program distinguishes cortical gray matter, 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 white matter lesions (WMLs) and infarction volumes. Total brain volume was calculated by summing the volumes of gray and white matter and, if present, the volumes of WMLs 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 WMLs adjacent to or within one centimeter 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 WMLs.

*Brain parameters*

Two brain parameters were used for analyses: WMLs, which was the sum of periventricular and deep WMLs in milliliters, expressed as percentage of ICV and brain parenchymal fraction (BPF), which was determined by calculating the total brain volume in milliliters as percentage of ICV. By taking percentages of ICV instead of crude brain parameters, correction for differences in head size was applied.

*Microvesicle measurements*

Complete measurement methods are described elsewhere [7]. MVs were isolated using ExoQuick™ (SBI) according to the manufacturer's protocol. Briefly, 150 µl EDTA plasma was centrifuged for 15 minutes at 3000 g. The supernatant was filtered over a 0.45 µm Spin-X filter (Corning),which was flushed with preheated PBS (37°C) and 38 µl 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 1500 g for 30 minutes at room temperature. After removing the supernatant, the pellet was lysed in 100 µl Roche Complete Lysis-M with protease inhibitors (EDTA free). Subsequently, the sample was filtered over a 0.22 µm 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 20x with Roche complete Lysis-M buffer. 50 µl of this diluted sample was analyzed in a multiplex immuno assay on levels of Cystatin C, Serpin G1, Serpin F2 & CD14, using a Bio-Rad Bioplex 200 system as described before [29]. Capture antibody, biotinylated detection antibody and antigen of all 4 proteins were purchased from R&D 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 MV protein level in pg/ml and WMLs in % or BPF in %. 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 microvesicle protein levels and measured structural brain changes.

To fulfill linear regression assumptions, WML volumes were naturally log-transformed as they showed skewed distributions. In order to directly compare strengths of associations, linear regressions were performed per standard deviation (SD) increase in MV protein level. Secondly, we assessed the prospective relationship between baseline MV protein levels and progression in brain parameters on MRI. This was done by relating baseline MV 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. Results are expressed as linear regression coefficients and 95% confidence intervals.

## Results

*Baseline characteristics*

Patient characteristics are displayed in Table 1. Seventy-nine percent of the initial patients were men and the mean age was 59±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±0.4 years, 534 patients underwent a second MRI of the brain. This follow-up cohort comprised 80% men and a mean age of 58±9 years. For comparisons of the two groups, we refer to supplemental Table 1.

*Cross-sectional association between MV protein levels and WMLs or BPF*

In the fully adjusted model, MV-Cystatin C and MV-CD14 were associated with volume of WMLs. Regression coefficients per SD MV-protein were 0.10 (95%CI 0.04-0.17) for MV-Cystatin C and 0.14 (95%CI 0.07–0.20) for MV-CD14 (Table 2). Also, MV-CD14 was associated with a statistically significantly lower BPF (regression coefficient per SD MV-CD14 -0.14 (95%CI -0.27–-0.01)); (Table 3). SD increases of MV-Serpin G1 and MV-Serpin F2 were not related with WMLs 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 MV protein levels and of WMLs or BPF*

In the fully adjusted model, MV-CD14 was associated with increase of WML volume (regression coefficient = 0.11 (95%CI 0.04-0.18)) as is displayed in Table 2. MV-Cystatin C, MV-Serpin G1 and MV-Serpin F2 were not related with increase of WMLs or BPF.

**Discussion**

In patients with various clinical manifestations of vascular disease, MV protein levels of Cystatin C and CD14 in plasma are associated with larger WMLs and more brain atrophy. Furthermore, higher MV-CD 14 levels are associated with an increase in WMLs 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 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 study [33], 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 WMLs [36] in large population-based cohort studies. In WMLs, the Cystatin C expression is upregulated in astrocytes, presumably due to a self-defense response in the process of white matter degeneration [37] Our results, based upon *MV*-Cystatin C, show similar results as increasing MV-Cystatin C is related to increased volume of WMLs. Cystatin C is secreted by all human tissues [38], which presumably concomitantly shed Cystatin $C^+$ MVs in order to directly influence more distant processes.

CD14 is a cofactor for Toll-like receptor 4 (TLR4), which regulates NFκB expression, indicating a role for CD14 in inflammation [39]. CD14 is expressed on monocytes [40], and it is therefore assumable that $CD14^+$ MVs originate from these monocytes. In our study, MV-CD14 levels were related to the volume and increase of WMLs. This indicates a MV-initiated inflammatory response in dealing with WMLs. These findings do not stand alone as several inflammatory biomarkers such as lipoprotein-associated phospholipase A2 (Lp-PLA2) and myeloperoxidase (MPO), have been associated with WMLs [41]. Although the effects of MV-CD14 on WMLs 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 MV-CD14 were inversely related to BPF at baseline in our study. MV-Serpin G1 (C1-inhibitor) and MV-Serpin F2 (α2-antiplasmin) were not associated with WMLs or BPF in our study, suggesting no substantial role of (inhibited) fibrinolysis in the pathophysiology surrounding WMLs or brain atrophy. To our understanding this is the first report on these Serpins and their potential role in the etiology of WMLs and brain atrophy. Whether and how MVs or MV protein levels might directly influence ischemic white matter lesions or brain volume is not known. Several mechanisms are proposed. We assume that MVs have blood-brain barrier penetrating properties, based upon recent data regarding exosomes [24, 25]. It could also be that MVs interact with endothelial cells through receptor-mediated endocytosis. Once fused with these endothelial cells, microvesicle protein levels might activate inflammation cascade signaling, leading to vascular damage and subsequent arterial stiffness. It might also activate hemostasis cascade signaling leading to the formation of (micro)thrombi. Alternatively, through endocytosis by endothelial cells, MV protein levels might influence the structural integrity of the endothelial layer resulting in greater BBB permeability. Neurodegenerative disorders as Alzheimer disease [43] and multiple sclerosis [44] have been associated with BBB integrity loss. The mechanism behind this loss is unclear and perhaps microvesicles play a role within this process. Future work will have to test these hypotheses. If true causation between MV protein levels and brain changes would be the case, strategies on influencing MV protein levels should be developed.

Strengths of the present prospective cohort study include the relatively large sample size with various MV 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 scales [45] 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 dilution of associations. Secondly, only 4 of the numerous proteins on the surface or within MVs were measured. It could well that besides these vascular risk markers, various other MV proteins have their etiology in the structural brain changes. Thirdly, we assume that plasma MVs 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

WMLs and BPF. Unfortunately no data is available whether these MVs are indeed present within the brain and it would be interesting to know if these MV proteins are present in cerebrospinal fluid and have a non-cerebral origin. If these MVs can truly pass the BBB and influence 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, MV-Cystatin C and MV-CD14 levels are related with the volume of WMLs and with BPF on brain MRI. MV-CD14 levels are related with an increase in the total volume of WMLs during follow-up. Microvesicle proteins levels may be causally related to brain changes in patients with clinically manifest vascular disease.

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**Disclosures**

The authors declare no conflict of interest.

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**Table 1: 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/m²)* | 26.9 ± 3.8 | 26.9 ± 3.6 | 26.8 ± 4.1 |
| Waist circumference (cm)* | 95 ± 11 | 95 ± 11 | 96 ± 12 |
| Blood pressure (mmHg)* | | | |
| 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 m²)* | 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) |
| Packyears 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) |

Values are expressed as: *Mean ± standard deviation, ‡Median (interquartile range).
†Defined according to the National Cholesterol Education Program ATPIII-revised guidelines.

**Table 2: Microvesicle protein levels and white matter lesions (WML)**

| | Model | WML | |
|---|---|---|---|
| | | WML at baseline n=994 | WML at follow-up* n= 534 |
| MV-Cystatin C (pg/µg) | 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) |
| MV-Serpin G1 (pg/µg) | 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) |
| MV-Serpin F2 (pg/µg) | 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) |
| MV-CD 14 (pg/µg) | 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)** |

Regression coefficients: increase in WML (log%) per 1SD increase in MV 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, packyears smoking and prevalent type 2 diabetes mellitus
*Models include additional adjustment for baseline WML and follow-up time

**Table 3: Microvesicle protein levels and Brain Parenchymal Fraction (BPF)**

| | Model | BPF | |
|---|---|---|---|
| | | BPF at baseline n=994 | BPF at follow-up* n= 534 |
| MV-Cystatin C (pg/µg) | 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) |
| MV-Serpin G1 (pg/µg) | 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) |
| MV-Serpin F2 (pg/µg) | 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) |
| MV-CD 14 (pg/µg) | 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) |

Regression coefficients: increase in BPF (%) per 1SD increase in MV 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, packyears smoking and prevalent type 2 diabetes mellitus
*Models include additional adjustment for baseline WML and follow-up time

**Suppl. Table 1.** Baseline characteristics and mean difference between follow-up and non-follow up patients

| | Follow-up patients N = 534 | Non-FU patients N = 460 | Mean difference (95% CI) |
|---|---|---|---|
| Age (years)* | 58 ± 9 | 60 ± 11 | 2.4 (1.2–3.6) |
| Male gender, n (%) | 426 (80) | 358 (78) | -0.02 (-0.07–0.03) |
| Body mass index (kg/m²)* | 26.9 ± 3.6 | 26.8 ± 4.1 | 0.2 (-0.6–0.4) |
| Waist circumference (cm)* | 95 ± 11 | 96 ± 12 | 0.7 (-0.7–2.0) |
| Blood pressure (mmHg)* | | | |
| Systolic | 142 ± 20 | 145 ± 23 | 1.4 (1.3–6.8) |
| Diastolic | 82 ± 11 | 83 ± 12 | 1.8 (0.4–3.2) |
| LDL-cholesterol (mmol/L)* | 2.8 ± 0.90 | 2.9 ± 0.97 | 0.06 (0.05–0.28) |
| HDL-cholesterol (mmol/L)* | 1.31 ± 0.38 | 1.28 ± 0.37 | 0.03 (-0.07–0.02) |
| Triglycerides (mmol/L)‡ | 1.41 (1.05 – 2.07) | 1.47 (1.09-2.07) | 0.07 (0.03–0.30) |
| Glucose (mmol/L)* | 6.2 ± 1.8 | 6.4 ± 1.9 | 0.12 (-0.02–0.45) |
| HsCRP (mmol/L)‡ | 1.6 (0.8 – 3.5) | 1.8 (0.9 - 3.8) | 0.60 (-0.80–1.57) |
| eGFR (ml/min/1.73 m²)* | 79.7 ± 17.1 | 75.4 ± 18.6 | 1.13 (-6.43–-2.00) |
| Homocysteine (µmol/L)* | 13.5 ± 4.9 | 14.6 ± 7.0 | 0.38 (0.38–1.88) |
| Prevalent type 2 diabetes, n (%) | 71 (13) | 85 (19) | -0.05 (-0.10–-0.00) |
| Metabolic syndrome, n (%)† | 184 (35) | 185 (40) | -0.06 (-0.12–0.01) |
| Smoking, n (%) | | | |
| Never | 100 (18) | 70 (15) | 0.04 (-0.01–0.08) |
| Ever | 257 (48) | 197 (43) | 0.05 (-0.01–0.12) |
| Current | 177 (33) | 193 (42) | -0.09 (-0.15–-0.03) |
| Packyears smoking* | 23.0 ± 20.1 | 23.1 ± 21.0 | 1.31 (-2.51–2.62) |
| History of vascular disease, n (%) | | | |
| Cerebrovascular disease | 144 (27) | 117 (25) | 0.02 (-0.04–0.07) |
| Coronary artery disease | 325 (61) | 255 (55) | 0.05 (-0.01–0.12) |
| Peripheral artery disease | 108 (20) | 133 (29) | -0.09 (-0.14–-0.03) |
| Aneurysm of the abdominal aorta | 37 (7) | 62 (14) | -0.07 (-0.10–-0.03) |
| Medication, n (%) | | | |
| Platelet-aggregation inhibitors | 407 (76) | 333 (72) | 0.04 (-0.02–0.09) |
| Blood pressure-lowering agents | 382 (72) | 320 (70) | 0.02 9-0.04–0.08) |
| Lipid-lowering agents | 388 (73) | 294 (64) | 0.09 (0.03–0.14) |
| Oral anticoagulants | 37 (7) | 43 (9) | -0.02 (-0.06–0.01) |

Values are expressed as: *Mean ± standard deviation, ‡Median (interquartile range).
†Defined according to the National Cholesterol Education Program ATPIII-revised guidelines.

STROBE Statement—checklist of items that should be included in reports of observational studies

| | Item No | Recommendation |
|---|---|---|
| **Title and abstract** | 1 | (*a*) Indicate the study's design with a commonly used term in the title or the abstract →Title/Abstract |
| | | (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found → Abstract |
| **Introduction** | | |
| Background/rationale | 2 | Explain the scientific background and rationale for the investigation being reported → Introduction page 3 |
| Objectives | 3 | State specific objectives, including any prespecified hypotheses → Introduction page 4 |
| **Methods** | | |
| Study design | 4 | Present key elements of study design early in the paper → Methods page 4 |
| Setting | 5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection → Methods page 4-5 |
| Participants | 6 | (*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up → Methods page 4-5<br>*Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls<br>*Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants |
| | | (*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed<br>*Case-control study*—For matched studies, give matching criteria and the number of controls per case |
| Variables | 7 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable → Methods page 5-6 |
| Data sources/ measurement | 8* | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group → Methods page 5-6 |
| Bias | 9 | Describe any efforts to address potential sources of bias → Methods page 6-7 |
| Study size | 10 | Explain how the study size was arrived at → Methods page 4-5 |
| Quantitative variables | 11 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why → Methods Page 6 |
| Statistical methods | 12 | (*a*) Describe all statistical methods, including those used to control for confounding → Methods page 7 |
| | | (*b*) Describe any methods used to examine subgroups and interactions → Methods page 7 |
| | | (*c*) Explain how missing data were addressed → Methods page 4 |
| | | (*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed → Methods page 5<br>*Case-control study*—If applicable, explain how matching of cases and controls was addressed<br>*Cross-sectional study*—If applicable, describe analytical methods taking account of |

| | |
|---|---|
| | sampling strategy |
| | (*e*) Describe any sensitivity analyses |

Continued on next page

| | | |
|---|---|---|
| **Results** | | |
| Participants | 13* | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed → Methods page 4, Results: Table 1 |
| | | (b) Give reasons for non-participation at each stage → Discussion |
| | | (c) Consider use of a flow diagram |
| Descriptive data | 14* | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders → Results: Table 1; page 7 |
| | | (b) Indicate number of participants with missing data for each variable of interest → N.A |
| | | (c) *Cohort study*—Summarise follow-up time (eg, average and total amount) |
| Outcome data | 15* | *Cohort study*—Report numbers of outcome events or summary measures over time → Results: Table 2; page 7-8 |
| | | *Case-control study*—Report numbers in each exposure category, or summary measures of exposure |
| | | *Cross-sectional study*—Report numbers of outcome events or summary measures |
| Main results | 16 | (*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included → Results: Table 2, page 7-8 |
| | | (*b*) Report category boundaries when continuous variables were categorized |
| | | (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period |
| Other analyses | 17 | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses → Results page 7; suppl Table 1 |
| **Discussion** | | |
| Key results | 18 | Summarise key results with reference to study objectives → Discussion page 8 |
| Limitations | 19 | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias → Discussion page 9 |
| Interpretation | 20 | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence → Discussion page 10 |
| Generalisability | 21 | Discuss the generalisability (external validity) of the study results → Discussion page 10 |
| **Other information** | | |
| Funding | 22 | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based → Funding sources page 10 |

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

**Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

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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| | |

# 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 4 previously identified EV proteins (Cystatin C, Serpin G1, Serpin F2 and CD14) are associated with cerebral white matter lesions (WML) and brain atrophy.

*Design*

Cohort study; cross-sectional and prospective

*Setting*

Single center, secondary and tertiary setting

*Participants*

1309 Patients with manifest vascular disease from the Second Manifestations of ARTerial disease-Magnetic Resonance (SMART-MR) study, of which 994 had successful brain Magnetic Resonance Imaging (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 relation 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 Tesla brain MRI was assessed with multivariable linear regression modeling. Subsequently, the relation between baseline EV protein levels and progression of atrophy and WML was analyzed 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% confidence interval) 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 white matter lesions (0.11 log %/SD (0.04–0.18). No relations with EV-Serpins were observed at baseline or at follow-up.

*Conclusions*

Extracellular vesicle proteins Cystatin C and CD14 are related to cerebral white matter lesions and the progression of brain atrophy in patients with manifest vascular disease, potentially identifying EVs in the etiology of structural brain changes.

**Article summary**

Article focus

- Assess the relationship between specific extracellular vesicle protein levels and structural brain changes
- Clinically important domain of patients with vascular disease
- Etiologic research

Key Messages

- Extracellular vesicle protein levels are related to cerebral white matter lesions and development of brain atrophy
- Analogous to vascular disease, extracellular vesicles and their protein levels could be causally related to brain changes, potentially identifying a promising novel source of biomarkers in the development of brain changes

Strengths and limitations

- 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 morphologic changes in the small vessels of the brain, characterized by white matter lesions (WML) on magnetic resonance imaging (MRI) [1]. The exact underlying pathophysiological mechanism regarding WML remains unclear. WML have been associated with cognitive decline [2] 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, dyslipidemia 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 depends 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 internalization with target cells [13]. For example, monocyte-derived EVs are internalized by endothelial cells and activate the nuclear factor-κB 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 subjects [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 neurons 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 stiffness [19], 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 hypothesized 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 relation between levels of these 4 EV proteins extracted from plasma, and brain parenchymal fraction (BPF) and white matter lesions (WML) 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-Magnetic Resonance (SMART-MR) study, a prospective cohort study aimed at investigating brain changes on magnetic resonance imaging (MRI) in 1,309 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 ischemic stroke, transient ischemic attack or amaurosis fugax. CAD was defined as a recent diagnosis of angina pectoris, myocardial infarction or coronary revascularization (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 abdominal aortic aneurysm of ≥3.0 centimeter 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 1 visit. After inclusion, patients underwent a standardized 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 participants still alive (n = 1,238) 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. The SMART-MR study was approved by the ethics committee of the University Medical Center Utrecht, and written informed consent was obtained from all participants.

*Magnetic resonance imaging protocol*

MR investigations were performed on a 1.5-Tesla whole-body system (Gyroscan 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 ms 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, 4.0 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 program distinguishes cortical gray 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 white matter lesions and infarction volumes. Total brain volume was calculated by summing the volumes of gray 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 WML were defined as WML adjacent to or within one centimeter of the lateral ventricles. Deep WML 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 milliliters, expressed as percentage of ICV and brain parenchymal fraction (BPF), which was determined by calculating the total brain volume in milliliters as percentage of ICV. By taking percentages of ICV instead of crude brain parameters, correction for differences in head size was applied.

*Extracellularvesicle measurements*

Complete measurement methods are described elsewhere [7]. EVs were isolated using ExoQuick™ (SBI) according to the manufacturer's protocol. Briefly, 150 µl EDTA plasma was centrifuged for 15 minutes at 3000 g. The supernatant was filtered over a 0.45 µm Spin-X filter (Corning),which was flushed with preheated PBS (37°C) and 38 µl 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 1500 g for 30 minutes at room temperature. After removing the supernatant, the pellet was lysed in 100 µl Roche Complete Lysis-M with protease inhibitors (EDTA free). Subsequently, the sample was filtered over a 0.22 µm 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 20x with Roche complete Lysis-M buffer. 50 µl of this diluted sample was analyzed in a multiplex immuno assay on levels of Cystatin C, Serpin G1, Serpin F2 & CD14, using a Bio-Rad Bioplex 200 system as described before [29]. Capture antibody, biotinylated detection antibody and antigen of all 4 proteins were purchased from R&D 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 % or BPF in %. 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 fulfill linear regression assumptions, WML volumes were naturally log-transformed as they showed skewed distributions. In order to directly compare strengths of associations, linear regressions were performed per standard deviation (SD) increase in EV protein level. Secondly, we assessed the prospective relationship between baseline EV protein levels and progression in brain parameters on MRI. This was done 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. Results are expressed as linear regression coefficients and 95% confidence intervals.

## Results

*Baseline characteristics*

Patient characteristics are displayed in Table 1. Seventy-nine percent of the initial patients were men and the mean age was 59±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±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±9 years, which corresponded with a mean follow-up age of 62±9 years. For the baseline comparisons of the two groups, we refer to supplemental Table 1.

*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 0.04-0.17) for EV-Cystatin C and 0.14 (95%CI 0.07–0.20) 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–-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 of WML or BPF*

In the fully adjusted model, higher EV-CD14 was associated with increase of WML volume (regression coefficient = 0.11 (95%CI 0.04-0.18)) 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.

## 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-CD 14 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 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 study [33], 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 WML [36] in large population-based cohort studies. In WML, the Cystatin C expression is upregulated in astrocytes, presumably due to a self-defense response in the process of white matter degeneration [37] Our results, based upon *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 tissues [38], which presumably concomitantly shed Cystatin $C^+$ EVs in order to directly influence more distant processes.

CD14 is a cofactor for Toll-like receptor 4 (TLR4), which regulates NFκB expression, indicating a role for CD14 in inflammation [39]. CD14 is expressed on monocytes [40], 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 a EV-initiated inflammatory response in dealing with WML. These findings do not stand alone as several inflammatory biomarkers such as lipoprotein-associated phospholipase A2 (Lp-PLA2) 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 (α2-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 etiology of WML and brain atrophy. Whether and how EVs or EV protein levels might directly influence ischemic white matter lesions or brain volume is not known. Several mechanisms are proposed. We assume that EVs have blood-brain barrier penetrating properties, based upon 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 cascade signaling, leading to vascular damage and subsequent arterial stiffness. It might also activate hemostasis cascade signaling 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 greater BBB permeability. Neurodegenerative disorders as Alzheimer disease [43] and multiple sclerosis [44] 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.

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 scales [45] 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 dilution of associations. Secondly, only 4 of the numerous proteins on the surface or within EVs were measured. It could well that besides these vascular risk markers, various other EV proteins have their etiology in the structural brain changes. Thirdly, 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 is available whether these EVs are indeed present within the brain and it would be interesting to know if these EV proteins are present in cerebrospinal fluid and have a non-cerebral origin. If these EVs can truly pass the BBB and influence 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 brain MRI. EV-CD14 levels are related with an increase in the total volume of WML during follow-up. Extracellular vesicle proteins levels may be causally related to brain changes in patients with clinically manifest vascular disease.

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**Contributorship Statement**

•Conception and design, or analysis and interpretation of data: DAK, LJK, CSPMU, FLJV
•Drafting the article or revising it critically for important intellectual content: DAK, DPVdK, LJK, CSPMU, YvdG, GP, MIG, FLJV
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**Disclosures**

The authors declare no conflict of interest.

**Data Sharing Statement**

Additional unpublished data from the SMART-study in general are available to all members of the SMART study group.

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**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/m²)* | 26.9 ± 3.8 | 26.9 ± 3.6 | 26.8 ± 4.1 |
| Waist circumference (cm)* | 95 ± 11 | 95 ± 11 | 96 ± 12 |
| Blood pressure (mmHg)* | | | |
| 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 m²)* | 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) |
| Packyears 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) |

Values are expressed as: *Mean ± standard deviation, ‡Median (interquartile range).
†Defined according to the National Cholesterol Education Program ATPIII-revised guidelines.

**Table 2: Extracellular vesicle protein levels and white matter lesions (WML)**

| | Model | WML | |
|---|---|---|---|
| | | WML at baseline n=994 | WML at follow-up* n= 534 |
| EV-Cystatin C (pg/µg) | 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/µg) | 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/µg) | 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/µg) | 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)** |

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, packyears smoking and prevalent type 2 diabetes mellitus
*Models include additional adjustment for baseline WML and follow-up time

**Table 3: Extracellular vesicle protein levels and Brain Parenchymal Fraction (BPF)**

| | Model | BPF | |
|---|---|---|---|
| | | BPF at baseline n=994 | BPF at follow-up* n= 534 |
| EV-Cystatin C (pg/µg) | 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/µg) | 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/µg) | 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/µg) | 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) |

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, packyears smoking and prevalent type 2 diabetes mellitus
*Models include additional adjustment for baseline WML and follow-up time

# ~~Microvesicle~~ 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*

~~Microvesicles~~ Extracellular vesicles (~~MV~~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 4 previously identified ~~MV~~EV proteins (Cystatin C, Serpin G1, Serpin F2 and CD14) are associated with cerebral white matter lesions (WML) and brain atrophy.

*Design*

Cohort study; cross-sectional and prospective

*Setting*

Single center, secondary and tertiary setting

*Participants*

1309 Patients with manifest vascular disease from the Second Manifestations of ARTerial disease-Magnetic Resonance (SMART-MR) study, of which 994 had successful brain Magnetic Resonance Imaging (MRI) and ~~MV~~EV protein level measurements

*Outcomes*

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

*Statistical methods*

The relation between ~~MV~~EV protein levels and WML volume (expressed as log transformed percentage of intracranial volume) and BPF (expressed percentage of intracranial volume) on 1.5 Tesla brain MRI was assessed with multivariable linear regression modeling. Subsequently, the relation between baseline ~~MV~~EV protein levels and progression of atrophy and WML was analyzed in 534 patients, in whom a follow-up MRI was obtained after 4 years.

*Results*

Higher ~~MV~~EV-Cystatin C and ~~MV~~EV-CD14 were significantly associated with larger WML volume (linear regression coefficient (95% confidence interval) 0.10 log %/SD (0.04 to 0.17) and 0.14 log %/SD (0.07 to 0.20), respectively. Higher ~~MV~~EV-CD14 was associated with more brain atrophy (-0.14 % /SD; -0.27 to -0.01). Baseline ~~MV~~EV-CD14 was significantly associated with increase of white matter lesions (0.11 log %/SD (0.04–0.18). No relations with ~~MV~~EV-Serpins were observed at baseline or at follow-up.

*Conclusions*

Extracellular ~~Micro~~vesicle proteins Cystatin C and CD14 are related to cerebral white matter lesions and the progression of brain atrophy in patients with manifest vascular disease, potentially identifying ~~MV~~EVs in the etiology of structural brain changes.

**Article summary**

Article focus

- Assess the relationship between specific extracellular vesicle protein levels and structural brain changes
- Clinically important domain of patients with vascular disease
- Etiologic research

Key Messages

- Extracellular vesicle protein levels are related to cerebral white matter lesions and development of brain atrophy
- Analogous to vascular disease, extracellular vesicles and their protein levels could be causally related to brain changes, potentially identifying a promising novel source of biomarkers in the development of brain changes

Strengths and limitations

- 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 morphologic changes in the small vessels of the brain, characterized by white matter lesions (WML) on magnetic resonance imaging (MRI) [1]. The exact underlying pathophysiological mechanism regarding WML remains unclear. WML have been associated with cognitive decline [2] 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, dyslipidemia and diabetes [5, 6].

Recently, extracellular ~~micro~~vesicles (~~MV~~EVs) have been identified as a novel, independent risk marker for the occurrence of vascular disease [7, 8]. ~~MV~~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]. ~~MV~~EVs are defined by size and antigen expression, which depends on their originating cell type [11,12]. Release of ~~MV~~EVs allows cells to influence (patho)physiological processes over distance in contrast to cell-cell contact.

~~MV~~EVs transfer proteins, mRNA, miRNA and bioactive lipids from one cell to another cell by either fusion or internalization with target cells [13]. For example, monocyte-derived ~~MV~~EVs are internalized by endothelial cells and activate the nuclear factor-κB pathway and expression of adhesion molecules on these endothelial cells, amplifying inflammation [14]. ~~MV~~EVs are procoagulant, as they carry phosphatidylserine on their membrane, which facilitates the assembly of components of the clotting cascade [15]. In addition, ~~MV~~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 ~~MV~~EVs in comparison with plasma of the same study subjects [17], suggesting an active role of ~~MV~~EVs in atherothrombotic disease rather than a trigger of disease onset.

Virtually every cell is capable of producing ~~MV~~EVs, including brain cells such as neurons and astrocytes [18]. Little is known about the role ~~MV~~EVs play in the development of brain atrophy or small vessel disease. However, microparticles, a subclass of ~~MV~~EVs, have been shown to influence arterial stiffness [19], 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 ~~MV~~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 hypothesized that ~~MV~~EV protein levels of Cystatin C, Serpin G1, Serpin F2 and CD14, in peripherally circulating plasma ~~MV~~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 relation between levels of these 4 ~~MV~~EV proteins extracted from plasma, and brain parenchymal fraction (BPF) and white

matter lesions (WML) 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-Magnetic Resonance (SMART-MR) study, a prospective cohort study aimed at investigating brain changes on magnetic resonance imaging (MRI) in 1,309 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 ischemic stroke, transient ischemic attack or amaurosis fugax. CAD was defined as a recent diagnosis of angina pectoris, myocardial infarction or coronary revascularization (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 abdominal aortic aneurysm of ≥3.0 centimeter 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 1 visit. After inclusion, patients underwent a standardized 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 ~~MV~~EV protein level measurement could be retrieved and had at least one ~~MV~~EV protein level measurement. Between January 2006 and May 2009, all participants still alive (n = 1,238) 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 ~~MV~~EV protein levels were available. The SMART-MR study was approved by the ethics committee of the University Medical Center Utrecht, and written informed consent was obtained from all participants.

*Magnetic resonance imaging protocol*

MR investigations were performed on a 1.5-Tesla whole-body system (Gyroscan 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 ms 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, 4.0 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 program distinguishes cortical gray 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 white matter lesions and infarction volumes. Total brain volume was calculated by summing the volumes of gray 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 WML were defined as WML adjacent to or within one centimeter of the lateral ventricles. Deep WML 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 milliliters, expressed as percentage of ICV and brain parenchymal fraction (BPF), which was determined by calculating the total brain volume in milliliters as percentage

of ICV. By taking percentages of ICV instead of crude brain parameters, correction for differences in head size was applied.

*Extracellular~~Micro~~vesicle measurements*

Complete measurement methods are described elsewhere [7]. ~~MV~~EVs were isolated using ExoQuick™ (SBI) according to the manufacturer's protocol. Briefly, 150 µl EDTA plasma was centrifuged for 15 minutes at 3000 g. The supernatant was filtered over a 0.45 µm Spin-X filter (Corning),which was flushed with preheated PBS (37°C) and 38 µl 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 1500 g for 30 minutes at room temperature. After removing the supernatant, the pellet was lysed in 100 µl Roche Complete Lysis-M with protease inhibitors (EDTA free). Subsequently, the sample was filtered over a 0.22 µm 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 20x with Roche complete Lysis-M buffer. 50 µl of this diluted sample was analyzed in a multiplex immuno assay on levels of Cystatin C, Serpin G1, Serpin F2 & CD14, using a Bio-Rad Bioplex 200 system as described before [29]. Capture antibody, biotinylated detection antibody and antigen of all 4 proteins were purchased from R&D 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 ~~MV~~EV protein level in pg/ml and WML in % or BPF in %. 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~~microvesicle~~ protein levels and measured structural brain changes.

To fulfill linear regression assumptions, WML volumes were naturally log-transformed as they showed skewed distributions. In order to directly compare strengths of associations, linear regressions were performed per standard deviation (SD) increase in ~~MV~~EV protein level. Secondly, we assessed the prospective relationship between baseline ~~MV~~EV protein levels and progression in brain parameters on MRI. This was done by relating baseline ~~MV~~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. Results are expressed as linear regression coefficients and 95% confidence intervals.

**Results**

*Baseline characteristics*

Patient characteristics are displayed in Table 1. Seventy-nine percent of the initial patients were men and the mean age was 59±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±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±9 years, which corresponded with a mean follow-up age of 62±9 years. For the baseline comparisons of the two groups, we refer to supplemental Table 1.

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

In the fully adjusted model, higher ~~MV~~EV-Cystatin C and ~~MV~~EV-CD14 were associated with more volume of WML. Regression coefficients per SD ~~MV~~EV-protein were 0.10 (95%CI 0.04-0.17) for ~~MV~~EV-Cystatin C and 0.14 (95%CI 0.07–0.20) for ~~MV~~EV-CD14 (Table 2). Also, higher ~~MV~~EV-CD14 was associated with a statistically significantly lower BPF (regression coefficient per SD ~~MV~~EV-CD14 -0.14 (95%CI -0.27–-0.01)); (Table 3). SD increases of ~~MV~~EV-Serpin G1 and ~~MV~~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 ~~MV~~EV protein levels and of WML or BPF*

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

**Discussion**

In patients with various clinical manifestations of vascular disease, ~~MV~~EV protein levels of Cystatin C and CD14 in plasma are associated with larger WML and more brain atrophy. Furthermore, higher ~~MV~~EV-CD 14 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 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 study [33], 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 WML [36] in large population-based cohort studies. In WML, the Cystatin C expression is upregulated in astrocytes, presumably due to a self-defense response in the process of white matter degeneration [37] Our results, based upon ~~MV~~*EV*-Cystatin C, show similar results as increasing ~~MV~~EV-Cystatin C is related to increased volume of WML. Cystatin C is secreted by all human tissues [38], which presumably concomitantly shed Cystatin $C^+$ ~~MV~~EVs in order to directly influence more distant processes.

CD14 is a cofactor for Toll-like receptor 4 (TLR4), which regulates NFκB expression, indicating a role for CD14 in inflammation [39]. CD14 is expressed on monocytes [40], and it is therefore assumable that $CD14^+$ ~~MV~~EVs originate from these monocytes. In our study, ~~MV~~EV-CD14 levels were related to the volume and increase of WML. This indicates a ~~MV~~EV-initiated inflammatory response in dealing with WML. These findings do not stand alone as several inflammatory biomarkers such as lipoprotein-associated phospholipase A2 (Lp-PLA2) and myeloperoxidase (MPO), have been associated with WML [41]. Although the effects of ~~MV~~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 ~~MV~~EV-CD14 were inversely related to BPF at baseline in our study.

~~MV~~EV-Serpin G1 (C1-inhibitor) and ~~MV~~EV-Serpin F2 (α2-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 etiology of WML and brain atrophy.

Whether and how ~~MV~~EVs or ~~MV~~EV protein levels might directly influence ischemic white matter lesions or brain volume is not known. Several mechanisms are proposed. We assume that ~~MV~~EVs have blood-brain barrier penetrating properties, based upon recent data regarding exosomes [24, 25]. It could also be that ~~MV~~EVs interact with endothelial cells through receptor-mediated endocytosis. Once fused with these endothelial cells, EV~~microvesicle~~ protein levels might activate inflammation cascade signaling, leading to vascular damage and subsequent arterial stiffness. It might also activate hemostasis cascade signaling leading to the formation of (micro)thrombi. Alternatively, through endocytosis by endothelial cells, ~~MV~~EV protein levels might influence the structural integrity of the endothelial layer resulting in greater BBB permeability. Neurodegenerative disorders as Alzheimer disease [43] and multiple sclerosis [44] have been associated with BBB integrity loss. The mechanism behind this loss is unclear and perhaps EV ~~micro~~vesicles play a role within this process. Future work will have to test these hypotheses. If true causation between ~~MV~~EV protein levels and brain changes would be the case, strategies on influencing ~~MV~~EV protein levels should be developed.

Strengths of the present prospective cohort study include the relatively large sample size with various ~~MV~~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 scales [45] 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 dilution of associations. Secondly, only 4 of the numerous proteins on the surface or within ~~MV~~EVs were measured. It could well that besides these vascular risk markers, various other ~~MV~~EV proteins have their etiology in the structural brain changes. Thirdly, we assume that plasma ~~MV~~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 is available whether these ~~MV~~EVs are indeed present within the brain and it would be interesting to know if these ~~MV~~EV proteins are present in cerebrospinal fluid and have a non-cerebral origin. If these ~~MV~~EVs can truly pass the BBB and influence 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, ~~MV~~EV-Cystatin C and ~~MV~~EV-CD14 levels are related with the volume of WML and with BPF on brain MRI. ~~MV~~EV-CD14 levels are related with an increase in the total volume of WML during follow-up. Extracellular ~~Micro~~vesicle proteins levels may be causally related to brain changes in patients with clinically manifest vascular disease.

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**Disclosures**

The authors declare no conflict of interest.

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**Table 1: Baseline ~~P~~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/m²)* | 26.9 ± 3.8 | 26.9 ± 3.6 | 26.8 ± 4.1 |
| Waist circumference (cm)* | 95 ± 11 | 95 ± 11 | 96 ± 12 |
| Blood pressure (mmHg)* | | | |
| 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 m²)* | 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) |
| Packyears 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) |

Values are expressed as: *Mean ± standard deviation, ‡Median (interquartile range).
†Defined according to the National Cholesterol Education Program ATPIII-revised guidelines.

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**Table 2: Extracellular ~~Micro~~vesicle protein levels and white matter lesions (WML)**

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

Regression coefficients: increase in WML (log%) per 1SD increase in ~~MV~~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, packyears smoking and prevalent type 2 diabetes mellitus
*Models include additional adjustment for baseline WML and follow-up time

**Table 3: Extracellular ~~Micro~~vesicle protein levels and Brain Parenchymal Fraction (BPF)**

| | | BPF | |
|---|---|---|---|
| | Model | BPF at baseline n=994 | BPF at follow-up* n= 534 |
| ~~MV~~EV-Cystatin C (pg/µg) | I | **-0.69 (-0.86 - -0.51)** | **-0.15 (-0.26 – -0.04)** |
| | II | -0.12 (-0.26 – 0.02) | -0.06 (-0.16 – 0.04) |
| *Mean 10.6* | III | -0.11 (-0.24 – 0.03) | -0.05 (-0.16 – 0.05) |
| *SD 5.3* | | | |
| ~~MV~~EV-Serpin G1 (pg/µg) | I | -0.00 (-0.18 – 0.17) | 0.04 (-0.05 – 0.14) |
| | II | 0.01 (-0.12 – 0.14) | 0.02 (-0.06 – 0.11) |
| *Mean 142.2* | III | 0.10 (-0.12 – 0.14) | 0.03 (-0.06 – 0.11) |
| *SD 85.6* | | | |
| ~~MV~~EV-Serpin F2 (pg/µg) | I | 0.05 (-0.12 – 0.23) | 0.06 (-0.04 – 0.16) |
| | II | -0.10 (-0.23 – 0.03) | -0.01 (-0.10 – 0.08) |
| *Mean 43.3* | III | -0.06 (-0.19 – 0.07) | 0.01 (-0.09 – 0.10) |
| *SD 30.4* | | | |
| ~~MV~~EV-CD 14 (pg/µg) | 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) |

Regression coefficients: increase in BPF (%) per 1SD increase in ~~MV~~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, packyears smoking and prevalent type 2 diabetes mellitus
*Models include additional adjustment for baseline WML and follow-up time

**Suppl. Table 1.** Baseline characteristics and mean difference between follow-up and non-follow up patients

| | Follow-up patients N = 534 | Non-FU patients N = 460 | Mean difference (95% CI) |
|---|---|---|---|
| Age (years)* | 58 ± 9 | 60 ± 11 | 2.4 (1.2–3.6) |
| Male gender, n (%) | 426 (80) | 358 (78) | -0.02 (-0.07–0.03) |
| Body mass index (kg/m²)* | 26.9 ± 3.6 | 26.8 ± 4.1 | 0.2 (-0.6–0.4) |
| Waist circumference (cm)* | 95 ± 11 | 96 ± 12 | 0.7 (-0.7–2.0) |
| Blood pressure (mmHg)* | | | |
| Systolic | 142 ± 20 | 145 ± 23 | 1.4 (1.3–6.8) |
| Diastolic | 82 ± 11 | 83 ± 12 | 1.8 (0.4–3.2) |
| LDL-cholesterol (mmol/L)* | 2.8 ± 0.90 | 2.9 ± 0.97 | 0.06 (0.05–0.28) |
| HDL-cholesterol (mmol/L)* | 1.31 ± 0.38 | 1.28 ± 0.37 | 0.03 (-0.07–0.02) |
| Triglycerides (mmol/L)‡ | 1.41 (1.05 – 2.07) | 1.47 (1.09-2.07) | 0.07 (0.03–0.30) |
| Glucose (mmol/L)* | 6.2 ± 1.8 | 6.4 ± 1.9 | 0.12 (-0.02–0.45) |
| HsCRP (mmol/L)‡ | 1.6 (0.8 – 3.5) | 1.8 (0.9 - 3.8) | 0.60 (-0.80–1.57) |
| eGFR (ml/min/1.73 m²)* | 79.7 ± 17.1 | 75.4 ± 18.6 | 1.13 (-6.43–-2.00) |
| Homocysteine (µmol/L)* | 13.5 ± 4.9 | 14.6 ± 7.0 | 0.38 (0.38–1.88) |
| Prevalent type 2 diabetes, n (%) | 71 (13) | 85 (19) | -0.05 (-0.10–-0.00) |
| Metabolic syndrome, n (%)† | 184 (35) | 185 (40) | -0.06 (-0.12–0.01) |
| Smoking, n (%) | | | |
| Never | 100 (18) | 70 (15) | 0.04 (-0.01–0.08) |
| Ever | 257 (48) | 197 (43) | 0.05 (-0.01–0.12) |
| Current | 177 (33) | 193 (42) | -0.09 (-0.15–-0.03) |
| Packyears smoking* | 23.0 ± 20.1 | 23.1 ± 21.0 | 1.31 (-2.51–2.62) |
| History of vascular disease, n (%) | | | |
| Cerebrovascular disease | 144 (27) | 117 (25) | 0.02 (-0.04–0.07) |
| Coronary artery disease | 325 (61) | 255 (55) | 0.05 (-0.01–0.12) |
| Peripheral artery disease | 108 (20) | 133 (29) | -0.09 (-0.14–-0.03) |
| Aneurysm of the abdominal aorta | 37 (7) | 62 (14) | -0.07 (-0.10–-0.03) |
| Medication, n (%) | | | |
| Platelet-aggregation inhibitors | 407 (76) | 333 (72) | 0.04 (-0.02–0.09) |
| Blood pressure-lowering agents | 382 (72) | 320 (70) | 0.02 9-0.04–0.08) |
| Lipid-lowering agents | 388 (73) | 294 (64) | 0.09 (0.03–0.14) |
| Oral anticoagulants | 37 (7) | 43 (9) | -0.02 (-0.06–0.01) |

Values are expressed as: *Mean ± standard deviation, ‡Median (interquartile range).
†Defined according to the National Cholesterol Education Program ATPIII-revised guidelines.

STROBE Statement—checklist of items that should be included in reports of observational studies

| | Item No | Recommendation |
|---|---|---|
| **Title and abstract** | 1 | (*a*) Indicate the study's design with a commonly used term in the title or the abstract →Title/Abstract |
| | | (*b*) Provide in the abstract an informative and balanced summary of what was done and what was found → Abstract |
| **Introduction** | | |
| Background/rationale | 2 | Explain the scientific background and rationale for the investigation being reported → Introduction page 3 |
| Objectives | 3 | State specific objectives, including any prespecified hypotheses → Introduction page 4 |
| **Methods** | | |
| Study design | 4 | Present key elements of study design early in the paper → Methods page 4 |
| Setting | 5 | Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection → Methods page 4-5 |
| Participants | 6 | (*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up → Methods page 4-5<br>*Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls<br>*Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants |
| | | (*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed<br>*Case-control study*—For matched studies, give matching criteria and the number of controls per case |
| Variables | 7 | Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable → Methods page 5-6 |
| Data sources/ measurement | 8* | For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group → Methods page 5-6 |
| Bias | 9 | Describe any efforts to address potential sources of bias → Methods page 6-7 |
| Study size | 10 | Explain how the study size was arrived at → Methods page 4-5 |
| Quantitative variables | 11 | Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why → Methods Page 6 |
| Statistical methods | 12 | (*a*) Describe all statistical methods, including those used to control for confounding → Methods page 7 |
| | | (*b*) Describe any methods used to examine subgroups and interactions → Methods page 7 |
| | | (*c*) Explain how missing data were addressed → Methods page 4 |
| | | (*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed → Methods page 5<br>*Case-control study*—If applicable, explain how matching of cases and controls was addressed<br>*Cross-sectional study*—If applicable, describe analytical methods taking account of |

| | |
|---|---|
| | sampling strategy |
| | (*e*) Describe any sensitivity analyses |

Continued on next page

| | | |
|---|---|---|
| **Results** | | |
| Participants | 13* | (a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed → Methods page 4, Results: Table 1 |
| | | (b) Give reasons for non-participation at each stage → Discussion |
| | | (c) Consider use of a flow diagram |
| Descriptive data | 14* | (a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders → Results: Table 1; page 7 |
| | | (b) Indicate number of participants with missing data for each variable of interest → N.A |
| | | (c) *Cohort study*—Summarise follow-up time (eg, average and total amount) |
| Outcome data | 15* | *Cohort study*—Report numbers of outcome events or summary measures over time → Results: Table 2; page 7-8 |
| | | *Case-control study*—Report numbers in each exposure category, or summary measures of exposure |
| | | *Cross-sectional study*—Report numbers of outcome events or summary measures |
| Main results | 16 | (*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included → Results: Table 2, page 7-8 |
| | | (*b*) Report category boundaries when continuous variables were categorized |
| | | (*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period |
| Other analyses | 17 | Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses → Results page 7; suppl Table 1 |
| **Discussion** | | |
| Key results | 18 | Summarise key results with reference to study objectives → Discussion page 8 |
| Limitations | 19 | Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias → Discussion page 9 |
| Interpretation | 20 | Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence → Discussion page 10 |
| Generalisability | 21 | Discuss the generalisability (external validity) of the study results → Discussion page 10 |
| **Other information** | | |
| Funding | 22 | Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based → Funding sources page 10 |

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

**Note:** An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.