PEER REVIEW HISTORY

BMJ Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form (see an example) and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below. Some articles will have been accepted based in part or entirely on reviews undertaken for other BMJ Group journals. These will be reproduced where possible.

ARTICLE DETAILS

TITLE (PROVISIONAL)	Extracellular vesicle protein levels are related to brain atrophy and cerebral white matter lesions in patients with manifest vascular disease: the SMART-MR study
AUTHORS	Kanhai, Danny; de Kleijn, Dominique; Kappelle, Jaap; Uiterwaal, Cuno; van der Graaf, Yolanda; Pasterkamp, Gerard; Geerlings, Mirjam; Visseren, Frank

VERSION 1 - REVIEW

REVIEWER	Professor Benjamin Aribisala University of Edinburgh United Kingdom
REVIEW RETURNED	29-Sep-2013

GENERAL COMMENTS	The authors studied associations between measures of microvesicles proteins (Cystatin C, Serpin G1, Serpin F2 and CD14), brain atrophy and white matter lesions (WML) in a large cohort using longitudinal data. At baseline, they found association between Systatin C & CD14 and WML. They also found association between CD14 and atrophy. However, only the association between CD14 and WML remained at the second time point after correcting for the first time point. This is a very interesting study which benefits from availability of longitudinal data hence allowing investigation of change over a period of time in the studied variables. The study design is very good and the statistical analysis was well carried out. There are a number of minor issues 1. In the results section of the abstracts, they mentioned that MV-Cystatin C and MV-CD14 were significantly associated with larger WML, what is the direction of MV, i.e. higher or lower? The same applies to the results section, most especially the last paragraph in the result section. 2. Line 2 of introduction defined white matter lesions as WML but they used WMLs immediately after that line. WMLs should be replaced by WML all through the manuscript because WML is already in a plural form. 3. Under the brain segmentation method, they segmented WML and infarct into different classes and separated from normal appearing white matter. In view of this, white matter should be replaced with normal appearing white matter. This will help the readers to know that they investigated only the normal tissue. This should be changed all through. 4. Table 1, Average age at baseline was 59 years, but almost 4 years after the average age reduced to 58 years. I think the age should increase moreso when the SD seems not to change. This does not look right or am I missing something? 5. Please add the volumetric measurement of WML and PFC to

	table 1.

REVIEWER	Paul Harrison University of Birmingham, United Kingdom
REVIEW RETURNED	10-Oct-2013

GENERAL COMMENTS	Kanhai et al have measured microvesicle protein levels and correlated Cystatin C and CD14 with white matter lesions and correlated these with progression of white brain atrophy I have a major problem with this paper in that the microvesicle isolation procedure uses the Exoquick reagent. The microvesicles isolated have not been fully characterised. The authors claim that their proteins are MV associated without characterising the preparations? The MV preparation is based upon utilisation of the Exoquick reagent which is designed to isolate exosomes and therefore is probably selecting only nanovesicles. The authors need to fully characterise the population of vesicles in terms of size, phenotype etc that have been isolated in their samples before they can claim that the proteins measured are MV associated.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1.
Name: Unknown
Institution and Country: Unknown

There are a number of minor issues
1. In the results section of the abstracts, they mentioned that MV-Cystatin C and MV-CD14 were significantly associated with larger WML, what is the direction of MV, i.e. higher or lower? The same applies to the results section, most especially the last paragraph in the result section.

To clarify directions of found associations, specifically direction of MV-proteins, “higher” has been added to the referred abstract and results sections. [Abstract, page 2; Results, page 8]

2. Line 2 of introduction defined white matter lesions as WML but they used WMLs immediately after that line. WMLs should be replaced by WML all through the manuscript because WML is already in a plural form.

We thank the reviewer for this correction. WMLs has been changed into WML throughout the manuscript.

3. Under the brain segmentation method, they segmented WML and infarct into different classes and separated from normal appearing white matter. In view of this, white matter should be replaced with normal appearing white matter. This will help the readers to know that they investigated only the normal tissue. This should be changed all through.

To our understanding there is a miscommunication here. Although the segmentation program differentiates between various tissues and structures, normal appearing white matter has only been

included in order to calculate total brain volume. The program separates normal appearing white matter from white matter lesions (WML), one of the outcomes of interest in this study. To clarify the distinction between normal appearing white matter and WML, the term white matter has been changed into normal appearing white matter in the “brain segmentation” paragraph on page 6.

[“This segmentation program distinguishes cortical gray matter, normal appearing white matter, sulcal and ventricular cerebrospinal fluid (CSF) and lesions…… 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.”]

4. Table 1, Average age at baseline was 59 years, but almost 4 years after the average age reduced to 58 years. I think the age should increase moreso when the SD seems not to change. This does not look right or am I missing something?

The reviewer is totally correct, the age at follow-up is higher. However, the ages supplied in the baseline table as well as the corresponding baseline characteristics paragraph (results, page 8) refer to age at baseline. It does not mention the age at follow-up. To clarify this the age at follow-up (62±9) is now also provided in this paragraph. [“..age of 58±9 years, which corresponded with a mean follow-up age of 62±9years.”] Results, page 7.
Additionally, the header of Table 1 “Patient characteristics” has been changed into “Baseline patient characteristics”.

5. Please add the volumetric of WML and PFC to table 1.

Ass suggested, the volumetric measurements of baseline WML and BPF are added to table 1. [Table 1, page 18]. Only the baseline values of WML and BPF are displayed as this is a baseline table.

Reviewer 2.
Name: Paul Harrison
Institution and Country: University of Birmingham, United Kingdom
Please state any competing interests or state ‘None declared’: None declared

Kanhai et al have measured microvesicle protein levels and correlated Cystatin C and CD14 with white matter lesions and correlated these with progression of white brain atrophy

Major points

The MV preparation is based upon utilization of the Exoquick reagent which is designed to isolate exosomes and therefore is probably selecting only nanovesicles. The authors need to fully characterise the population of vesicles in terms of size, phenotype etc that have been isolated in their samples before they can claim that the proteins measured are MV associated.

The reviewer is correct that the use Exoquick reagent suggests that solely exosomes have been isolated. However, due to the confusion of the nomenclature of all vesicles, especially at the time SBI brought the reagent on the market, this is incorrect. It is now clear that Exoquick isolates all vesicles and not only exosomes. To prevent further confusion, we have changed the name “microvesicle” that we have used in the manuscript into the more general name “extracellular vesicles” as suggested by the International Society for Extracellular Vesicles (ISEV). Next to this, we performed a nanocyte analysis on size of the Exoquick isolated vesicles (attached). This showed that the range in size of the

isolate plasma vesicles is from 20 to 300 nm with a mean around 100 nm. This surpasses the exosomes in size and confirms again that Exoquick precipitates not only Exosomes (theoretically between 50-100 nm) but also larger vesicles. Full isolation process as well as characteristics of the isolated vesicles are referred to [Kanhai et al. Int. J. Cardiol. 2013]. Floatation studies have been shown that the 4 proteins are associated with floating vesicles in the same article.

VERSION 2 – REVIEW

REVIEWER	Paul Harrison University of Birmingham, UK
REVIEW RETURNED	02-Dec-2013

- The reviewer completed the checklist but made no further comments.