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## Detection of the receptor for advanced glycation endproducts in neuronally-derived exosomes in plasma

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### Abstract

Exosomes are nanovesicles that participate in cell-to-cell communication and are secreted by a variety of cells including neurons. Recent studies suggest that neuronally-derived exosomes are detectable in plasma and that their contents likely reflect expression of various biomarkers in brain tissues. The receptor for advanced glycation endproducts (RAGE) has been implicated in the pathophysiology of Alzheimer's disease (AD) and is increased in brain regions affected by AD. The goal of our project was to determine whether RAGE is present in plasma exosomes, and specifically exosomes derived from neurons. Exosomes were isolated from plasma samples (n=8) by precipitation (ExoQuick) and ultracentrifugation methods. Neuronally-derived exosomes were isolated using a biotin-tagged L1 Cell Adhesion Molecule (L1CAM) specific antibody and streptavidin-tagged agarose resin. RAGE expression was measured by Western blotting and ELISA. Western Blotting showed that RAGE is present in L1 CAM-positive exosomes isolated using both methods. Mean (SD) exosomal RAGE levels were 164 (60) pg/ml by ExoQuick and were highly correlated with plasma sRAGE levels ( $r=0.87$ ,  $p=0.005$ ), which were approximately 7.5-fold higher than exosomal levels. Weak to moderate correlations were found between exosomal RAGE and age, BMI, and cognitive function. These results show for the first time that RAGE is present in neuronally-derived plasma exosomes, and suggest that exosomal RAGE may be novel biomarker that reflects pathophysiological processes in the brain.

### Keywords

receptor for advanced glycation endproducts; exosomes; cognition

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## INTRODUCTION

Over the last 10 years, accumulating evidence has emerged to support a key role for the receptor for AGEs (RAGE) in the pathophysiology of Alzheimer's disease (AD) [1, 2]. By binding to both advanced glycation endproducts and amyloid- $\beta$  (A $\beta$ ), RAGE contributes to the classical pathological features of AD by stimulating A $\beta$  production in the brain and regulating the influx of circulating A $\beta$  across the blood-brain barrier [3, 4]. RAGE also promotes senile plaque formation via tau hyperphosphorylation, synaptic dysfunction, and neuronal death [3, 5–7]. Soluble forms of RAGE (sRAGE) are generated by proteolytic cleavage of the membrane-bound receptor or by alternative splicing [8, 9]. In contrast to RAGE, sRAGE can prevent A $\beta$  transport into the brain and A $\beta$ -induced neurotoxicity [7, 10]. These discoveries have led to the design of randomized clinical trials testing the efficacy of RAGE inhibitors in the treatment of mild-to-moderate AD [1, 2].

Both neuropathological and epidemiological studies show that RAGE expression is increased in patients with mild cognitive impairment (MCI) and AD compared to cognitively normal controls, whereas sRAGE levels are lower [3, 11–17]. Moreover, lower sRAGE levels are associated with cognitive impairment [11], as well as reduced brain volumes and increased cerebrovascular disease [18]. As such, low sRAGE levels may reflect an impaired endogenous protective response; yet, plasma/serum levels of sRAGE are non-specific. Thus, a blood-based RAGE biomarker that specifically reflects brain pathology is needed in epidemiological research to better understand the role of RAGE in neurodegenerative diseases.

Exosomes are small (~30–150 nm) extracellular vesicles of endosomal origin that are released in both a constitutive and stimulated manner by a variety of cell types [19]. Importantly, exosomes can be released from neurons and detected in the blood, where their contents reflect the proteins, lipids, RNA, and other constituents of the parent cell. Given that exosomes may be able to cross the blood-brain barrier [20], measuring the contents of neuronally-derived exosomes found in blood has the potential to provide a brain “biopsy” that yields important information about pathophysiological processes occurring in the brain. Moreover, exosomes participate in cell-to-cell communication, removal of unwanted proteins, and transfer of pathogens (including prion-like misfolded proteins), mRNAs and miRNAs between cells, and consequently, are able to modulate protein synthesis and gene expression in recipient cells [21–23]. Previous studies indicate that neuronally-derived exosomes can be detected in plasma using L1 cell adhesion molecule (L1CAM), a surface protein located on neurons, and that their contents likely reflect protein expression in the brain [24–28]. Since exosomal RAGE may have important implications for AD, the purpose of this study was to determine whether RAGE is present in plasma exosomes, and specifically neuronally-derived exosomes.

## MATERIALS AND METHODS

### Study Population

Blood samples were collected from obese older adults who were recruited to participate in a weight loss study, but failed to meet all eligibility criteria (n=8). Individuals reported to the

laboratory in the morning after an overnight fast. Venous blood was drawn into EDTA tubes, centrifuged to collect the plasma fraction, and stored at  $-80^{\circ}\text{C}$  until analysis. The Montreal Cognitive Assessment (MoCA) was administered to assess global cognitive function [29]. MoCA scores were available for all but one participant. Written informed consent was provided prior to all data and blood collection.

### Exosome Isolation from Plasma

Exosomes were isolated from plasma samples using two different methods: an ExoQuick precipitation method and an ultracentrifugation method, as we have described previously [30–32]. For the ExoQuick method, 0.5 ml plasma was centrifuged at  $3000 \times g$  for 15 minutes to remove cellular debris and collect supernates. 100  $\mu\text{l}$  of ExoQuick Exosome Precipitation Solution (EXOQ; System Biosciences, Inc., Mountainview, CA) was added. Suspensions were incubated overnight and then centrifuged at  $1,500 \times g$  for 30 minutes. The supernatant from each sample was discarded and the remaining pellet was subjected to another centrifugation at  $1500 \times g$  for 5 minutes. All remaining trace fluid was removed, leaving a pellet containing exosomes. The exosome pellet was then suspended in lysis buffer for Western Blotting. For the ultracentrifugation method, one-half milliliter of plasma was gathered and subjected to serial centrifugations at  $500 \times g$  and  $2000 \times g$  for five minutes each. The samples were transferred to fresh vials and then centrifuged at  $10,000 \times g$  to remove microvesicles. Supernates were collected and filtered through 0.22  $\mu\text{m}$  filters. Afterwards, suspensions were ultracentrifuged at  $100,000 \times \text{rpm}$ . The resultant pellet, which contained exosomes, was then suspended in lysis buffer for Western Blotting.

### Isolation of Neuronally-derived Exosomes From Plasma

Neuronally-derived exosomes were isolated from plasma as previously described [33]. Briefly, 0.5 ml of plasma was incubated with 0.15 ml of thromboplastin-D (Fisher Scientific, Inc., Hanover Park, IL) at room temperature for 60 minutes, followed by addition of 0.35 ml of calcium- and magnesium-free Dulbecco's phosphate buffered saline (DPBS) with protease inhibitor cocktail and phosphatase inhibitor cocktail (Pierce Halt, Thermo Scientific, Inc., Rockford, IL). After centrifugation at  $1,500 \times g$  for 20 minutes, supernates were mixed with 252 ml of ExoQuick exosome precipitation solution and incubated for 1 hour at  $4^{\circ}\text{C}$ . Resultant exosome suspensions were centrifuged at  $1,500 \times g$  for 30 minutes at  $4^{\circ}\text{C}$  and each pellet was resuspended in 250 ml of DPBS with inhibitor cocktails. Each sample was incubated for 1 hour at  $4^{\circ}\text{C}$  with 1 mg of mouse anti-human CD171 (L1CAM) biotinylated antibody (clone 5G3, eBioscience, San Diego, CA) and then 25 ml of streptavidin-agarose resin (Thermo Scientific, Inc.) plus 50 ml of 3% bovine serum albumin (BSA; 1:3.33 dilution of Blocker BSA 10% solution in DPBS). After centrifugation at  $200 \times g$  for 10 minutes at  $4^{\circ}\text{C}$  and removal of the supernate, each pellet was suspended in 50  $\mu\text{l}$  of 0.05 M glycine-HCl (pH 3.0) by vortexing for 10 seconds. Each suspension then received 500  $\mu\text{l}$  of M-PER mammalian protein extraction reagent (Thermo Scientific, Inc., Rockford, IL) that had been adjusted to pH 8.0 with 1 M Tris-HCl (pH 8.6) and contained cocktail inhibitors. The suspensions were incubated at  $37^{\circ}\text{C}$  for 10 minutes, vortex-mixed for 15 seconds, and then stored at  $-80^{\circ}\text{C}$  until Western Blotting.

## Western Blotting

Total cell lysates were prepared using 1× RIPA buffer containing protease and phosphatase inhibitor cocktail solution. Approximately, 40–60 µg of protein lysate per sample was denatured in Laemmli sample buffer containing β-mercaptoethanol and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on Tris-glycine gel. The separated proteins were transferred on to a nitrocellulose membrane followed by blocking with 5% non-fat milk powder (w/v) in Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween 20) for 1 h at room temperature. Membranes were probed for RAGE protein using a rabbit polyclonal antibody corresponding to amino acids 39–58 of human RAGE (Abcam, Cambridge, UK), followed by the appropriate peroxidase-conjugated secondary antibody and then visualized by an electrochemiluminescence detection system.

## RAGE Assay

Total RAGE was measured in plasma and exosomes isolated from plasma using a commercially available ELISA that uses a monoclonal antibody raised against amino acids Gln24-Ala344 in the N-terminal extracellular domain of human RAGE (R&D Systems, Minneapolis, MN). This assay quantifies all circulating isoforms including cleaved isoforms shed into the bloodstream by extracellular metalloproteinases as well as secreted isoforms generated by alternative splicing.

## RESULTS

### Participant Characteristics

As shown in Table 1 the participants in this study were primarily older white females. All were obese and had normal cognitive function based on a cut-off score of 22 for the MoCA [34].

### RAGE is present in plasma exosomes

Western Blot results showed that RAGE was present in plasma exosomes isolated using both the precipitation and ultracentrifugation methods (Figure 1). Exosomal RAGE expression was confirmed by ELISA where mean (SD) RAGE levels were 1225 (618) pg/ml in plasma and 164 (60) pg/ml in plasma exosomes obtained via ExoQuick (Table 1). Although RAGE levels in plasma exosomes were approximately 13% of that found in the plasma fraction, plasma and exosomal RAGE levels were highly correlated ( $r=0.87$ ,  $p=0.005$ ). Moreover, the assay results were robust, with intra-assay coefficients of variation of <4%. Protein concentrations in the samples obtained via ultracentrifugation were very dilute and were generally below the minimum detectable dose for the RAGE ELISA (<16 pg/ml).

### RAGE is present in neuronally-derived plasma exosomes

Plasma samples from 4 participants were used to isolate neuronally-derived exosomes using an antibody to L1CAM. Western Blot results confirmed that RAGE is detectable in both L1CAM-positive (i.e., neuronally-derived) and L1CAM-negative exosomes (Figure 2). The results show that RAGE expression is relatively lower in L1CAM-positive exosomes, and highly variable, compared to L1CAM-negative exosomes.

### RAGE in plasma and plasma exosomes is correlated with cognitive function

A modest correlation was found between plasma sRAGE levels and MoCA score ( $r=0.40$ ,  $p=0.38$ , Figure 3). RAGE levels in plasma exosomes were not correlated with MoCA ( $r=0.07$ ,  $p=0.89$ ); however, when exosomal RAGE was normalized to the total protein content, the association was stronger ( $r=0.25$ ,  $p=0.58$ ). This finding may be related to the significant association between MoCA score and total exosomal protein ( $r=-0.79$ ,  $p=0.03$ ) (Figure 4), which ranged from 6.27  $\mu\text{g}/\mu\text{l}$  to 9.12  $\mu\text{g}/\mu\text{l}$ . RAGE levels in both plasma and plasma exosomes were similarly correlated with BMI:  $r=-0.73$ ,  $p=0.04$  and  $r=-0.71$ ,  $p=0.05$ , respectively. Weak to moderate correlations were also observed with age:  $r=-0.59$ ,  $p=0.12$  for plasma sRAGE and  $r=-0.28$ ,  $p=0.50$  for exosomal RAGE.

## DISCUSSION

This study reports for the first time the discovery of a novel source of RAGE in the blood - exosomes. We have confirmed (via Western blot and ELISA) that plasma exosomes contain RAGE protein. Because exosomes participate in cell-to-cell communication and mediate many cellular processes, the presence of RAGE in exosomes suggests that the removal and/or transfer of RAGE between cells has important biological relevance. The presence of RAGE in neuronally-derived plasma exosomes also highlights potential implications for the development and progression of neurodegenerative diseases. Notably, RAGE expression in neuronally-derived plasma exosomes appears to be highly variable. Future studies should investigate whether this variability correlates with the onset of AD.

As has been shown previously [11], we found a positive association between plasma sRAGE levels and MoCA. Plasma RAGE reflects soluble isoforms, suggesting that low sRAGE levels are a risk factor for or a biomarker of cognitive impairment. In contrast, we found no association between exosomal RAGE levels and cognition. Since the ExoQuick method lyses the exosomes, the analysis of exosomal RAGE by the present ELISA likely quantifies both membrane-bound RAGE on the surface of the exosome and sRAGE found within the vesicle. The fact that these isoforms have differing associations with AD could explain the lack of association between exosomal RAGE and MoCA score.

Interestingly, another novel finding was the strong correlation between exosomal protein content and MoCA score. AD is characterized by the misfolding and aggregation of key proteins, and exosomes are known to participate in the transfer of unwanted proteins and pathogens [21]. As such, it is possible that the inverse association between exosomal protein content and MoCA score reflects a higher burden of misfolded or damaged proteins in individuals with greater cognitive impairment and the subsequent packaging of these proteins into exosomes for transfer or removal.

Using an exosomal biomarker derived from neuronal sources has the potential to not only facilitate the study of relevant brain pathology and neurodegeneration in clinical populations, but also aid in AD risk prediction. Our study suggests that exosomal RAGE may be a useful biomarker and/or therapeutic target in this regard. However, utilizing exosomes in the clinical setting will require greater knowledge of the mechanisms of biogenesis and secretion, as well as the development of more accurate methods to isolate and characterize

exosomal fractions, including the origin and physiological function of different subpopulations [19]. Further research is needed to identify the exact RAGE isoforms present in exosomes (membrane-bound or soluble isoforms), determine where they are located (on the surface or within the vesicle), and better understand if and how trafficking of RAGE in plasma exosomes affects surrounding cells and contributes to pathophysiological processes.

In conclusion, we found that RAGE is detectable in plasma exosomes, and specifically exosomes derived from neurons. Despite the limitations of the current study (e.g., small sample size, non-specific RAGE assay), our findings contribute to the growing body of literature supporting a role for RAGE in the brain and in the pathogenesis of cognitive decline and dementia.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

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## ABBREVIATIONS

<b>AD</b>	Alzheimer's disease
<b>BMI</b>	body mass index
<b>L1CAM</b>	L1 cell adhesion molecule
<b>MCI</b>	Mild cognitive impairment
<b>MoCA</b>	Montreal Cognitive Assessment
<b>RAGE</b>	receptor for advanced glycation endproducts
<b>sRAGE</b>	soluble receptor for advanced glycation endproducts

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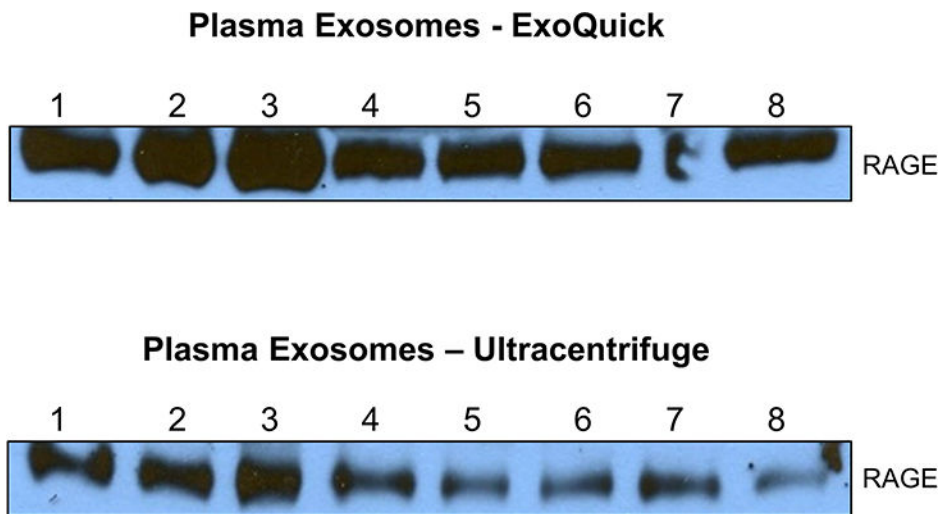
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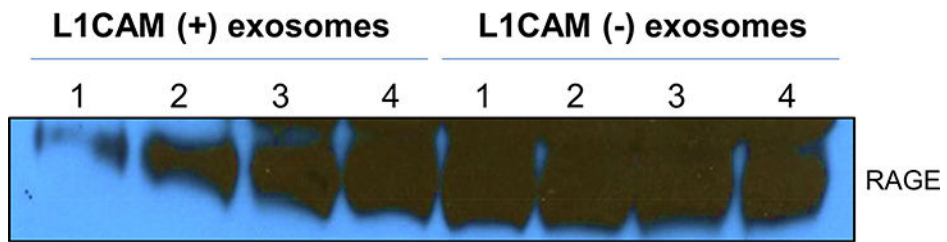


**HIGHLIGHTS**

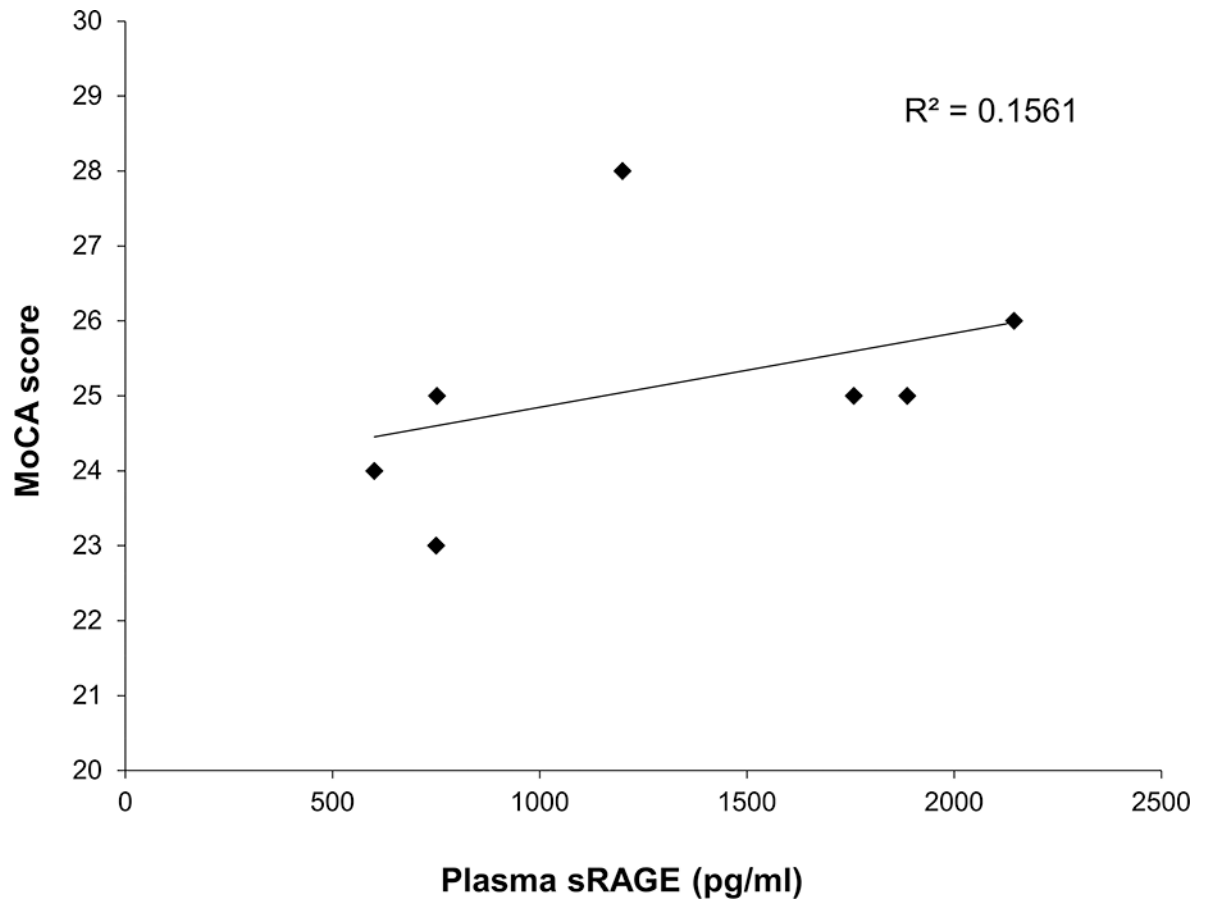
- RAGE is present in plasma exosomes
- RAGE is present in neuronally-derived exosomes in plasma
- Exosomal RAGE is weakly to moderately associated with age, BMI, and cognition
- Higher total protein content in plasma exosomes is associated with worse cognition



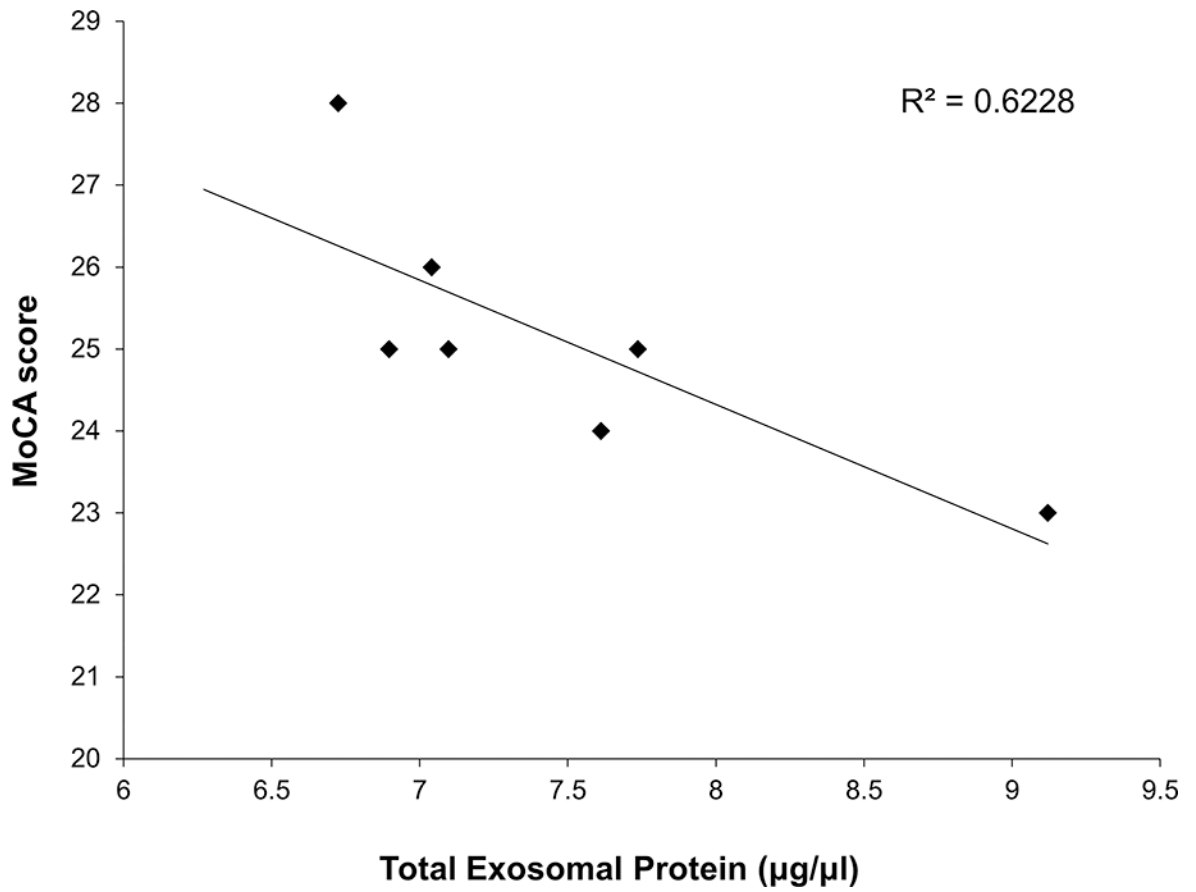
**Figure 1. RAGE is present in plasma exosomes.** Exosomes were isolated from plasma by ExoQuick precipitation method or by ultracentrifugation. Exosomes were lysed and analyzed for RAGE expression by Western blotting and representative blots are shown.



**Figure 2. RAGE is present in neuronally-derived exosomes from plasma.** L1CAM-positive and L1CAM-negative exosomes were isolated from plasma as detailed in the methods and analyzed for RAGE expression by Western blotting. A representative blot is shown.



**Figure 3.**  
Lower plasma sRAGE levels are associated with lower cognitive function.



**Figure 4.** Higher total exosomal protein content is associated with lower cognitive function.

**Table 1:**

Clinical characteristics of study participants (n=8)

<b>Clinical Characteristic</b>	<b>Mean <math>\pm</math> SD or N (%)</b>	<b>Range</b>
Age (yrs)	69.5 $\pm$ 4.1	65 – 77
Female gender	7 (87.5%)	--
White race	6 (75%)	--
BMI (kg/m <sup>2</sup> )	36.3 $\pm$ 3.7	31.0 – 42.3
MoCA	25.1 $\pm$ 1.6	23 – 28
Plasma sRAGE (pg/ml)	1225 $\pm$ 618	602 – 2145
Exosomal RAGE (pg/ml)	164 $\pm$ 60	93 – 248

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