



Circulating autoantibodies against the NR2 peptide of the NMDA receptor are associated with subclinical brain damage in hypertensive patients with other pre-existing conditions for vascular risk

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ABSTRACT

Arterial hypertension (HT) and other vascular pre-existing conditions (PEC) generate asymptomatic brain damage which increases the occurrence of stroke and cognitive decline. The aim of this work was to explore if serum antibodies against the NR2 subunit of the NMDA receptor (NR2Ab) could predict subclinical brain damage (SBD) in hypertensive patients with PEC. Forty seven neurologically asymptomatic hypertensive subjects were classified according to the number of PEC (retinopathy, overweight/obesity, diabetes mellitus and dyslipidemia). NR2A/B Ab were measured in serum employing an ELISA method. 3.0-T Brain MRI imaging was performed, and visual ratings of white matter hyperintensities (WMH) and counts of dilated Virchow-Robin spaces (DVRS) and lacunes were obtained. Brain atrophy was evaluated with cortical thickness measurements and linear measures. Higher levels of NR2Ab were associated with more severe periventricular WMH (PWMH), more DVRS and more severe SBD; while greater frontal interhemispheric fissure width (IHFV), as a linear measure of frontal atrophy, was inversely related with NR2Ab. Overall and regional cortical thickness were not significantly associated with NR2 Ab. A multivariate analyses showed that IHFV and PWMH were the only variables independently associated with serum NR2Ab concentration. ROC analysis revealed that NR2Ab (cutoff: 1.7 ng/ml) predicted PWMH with a sensitivity and specificity of 65% and 87% respectively.

Conclusions

Serum NR2Ab levels may reflect SBD in HT subjects with PEC, especially in younger populations at risk, where age-related cortical atrophy has not yet been fully established.

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1. Introduction

Subclinical brain lesions are often detected by neuroimaging in older asymptomatic patients, but they tend to occur earlier and ap-

Abbreviations: HT, arterial hypertension; PEC, vascular pre-existing conditions; SLI, silent lacunar infarcts; WMH, white matter hyperintensities; DVRS, dilated Virchow-Robin spaces; SBD, subclinical brain damage; IHFV, frontal interhemispheric fissure width

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pear to be more severe in patients with hypertension [1]. In addition to HT, the presence of pre-existing conditions (PEC) (diabetes, retinopathy, dyslipidemia, and overweight/obesity) in asymptomatic patients generates in the brain a chronic, sustained and low grade subclinical damage. This damage is characterized mainly by the appearance of silent lacunar infarcts (SLI), white matter hyperintensities (WMH) and dilated Virchow-Robin spaces (DVRS) [2–3]; whose presence has been shown to increase 2–3 folds the risk of stroke, mild cognitive impairment and dementia [4–5].

Screening for subclinical brain lesions in risk population in the community is rarely performed because it requires costly and scarce imaging techniques. Recently our group reported elevated serum neuron specific enolase (NSE) as a predictor of subclinical brain damage and of the subsequent occurrence of brain-related vascular events in hypertensive subjects [6], and proposed that raised serum levels of brain specific proteins could be a cost-effective approach [7].

NR2 is one of the subunits of the NMDA receptor, which is the major excitatory amino acid receptor in the central nervous system, and it has been previously described as a biomarker which correlates with prior isolated or multiple ischemic stroke and increased risk of near-term cerebral ischemic events in patients with PEC [8–11].

In this work we hypothesize that the concentration of autoantibodies against the NR2 subunit (NR2Ab) in blood could be associated with the presence of subclinical brain damage in hypertensive subjects with PEC. The main purpose was to carry out a pilot study in a health area of Havana to evaluate the potential utility of assessing circulating NR2 antibodies as a predictor of brain damage in neurologically asymptomatic hypertensive subjects with other comorbidities.

2. Materials and methods

2.1. Clinical data

Forty seven neurologically asymptomatic subjects with arterial hypertension were included from a health area of the Polyclinic “19 de Abril”. This health area - which assists a population of 522 adults - was the universe studied. Taking into account the exclusion criteria (previous neurological disease, overt vascular diseases, chronic degenerative or inflammatory diseases or malignancies), the eligible population came down to 468. Sample calculation was carried out employing the statistical package EPIDAT V3.1 for epidemiological analyses of tabulated data, considering the previous conditions and an estimated prevalence for arterial hypertension of 20%. With a 95% confidence level, a precision of 0.05 and an expected error of 0.05, the sample number obtained was 46. The diagnosis of hypertension was performed by LAC (family care physician assisting this health area, with a Master's degree in Cerebrovascular Diseases) taking into account the Eight Report on the Management of High Blood Pressure in Adults [12].

Mean age of the patients was 59.5 years (39–76), 60.8% were women. Eighty seven % of the patients were receiving antihypertensive medication. Duration of hypertension for the whole group was 14.5 ± 10.5 years (2–45 years), and 67.7% had been affected for > 5 years.

Systolic blood pressure (SBP), diastolic blood pressure (DBP) and grade of retinopathy were measured as previously described [13]. Mean \pm SD for SBP and DBP measurements before blood extraction were 130.8 ± 18.4 and 81.3 ± 10.0 mmHg, respectively. Fifty % of the patients displayed blood pressure readings above 139/89. Most patients (88.4%) had some degree of retinopathy: (GI: 32.6%; GII: 53.5% and GIII: 2.3%).

Pre-existing conditions (PEC) for vascular risk were registered in all subjects: retinopathy, overweight/obesity, diabetes mellitus (DM) and dyslipidemia. Criteria for obesity or overweight was body-mass index (BMI) > 25; dyslipidemia was considered when cholesterol > 6.0 mM and/or triglycerides > 1.2 mM. Hypertensive subjects were divided in two groups according to the number of vascular pre-existing conditions (PEC): one group with none or few PEC (from 0 to 2) and another with more PEC (from 3 to 4).

2.2. Blood sampling and analytical procedures

Five milliliters of venous blood were withdrawn from each subject into vacuum tubes with no additives. Samples were centrifuged and the separated serum was stored at -20°C for further processing. Autoantibodies directed against the NR2A/B peptides of the NMDA receptor were measured in serum employing an ELISA method: Gold Dot NR2 Antibody Assay kit (CIS Biotech, Inc., Atlanta, GA, USA). Serum samples, calibrators and controls were analyzed accord-

ing to the manufacturer's manual, and assays were run in duplicate. The manufacturers of the NR2Ab kit report a reference interval of 0.87–2.0 ng/ml in samples from 177 apparently healthy subjects (89 males and 88 females), in an age range of 40–80 years, and with the following ethnic composition: Caucassian-122; African American-51; Hispanic-1 and Asian-3 (www.cisbiotech.com). Nine supposedly healthy subjects were examined in our laboratory, although 8 of them were overweight (BMI > 25) and the mean age was lower than the patients' (42 vs 59.2 years). The median for NR2Ab levels in these control subjects was 1.97 (1.42–2.40) ng/ml.

Serum concentrations of cholesterol and triglycerides were measured employing spectrophotometric assay kits from CPM Scientifica Tecnologie Biomediche (Italy).

2.3. Imaging protocol

Brain MRI imaging was possible in thirty-two patients, who were studied by following a standard protocol. Scans were made on a 3.0-T scanner (Siemens, MAGNETOM Concerto, Germany). The protocol included the following sequences: axial T2-weighted (echo time 113 ms, repetition time 4.950, voxel size $1.2 \times 1 \times 1$ mm³, total slices 19); FLAIR axial (echo time 89 ms, repetition time 6.230, voxel size $1.2 \times 1 \times 1$ mm³, total slices 19), and T1 f3d coronal and sagittal sequences (echo time 8.4 ms, repetition time 20, voxel size $1.3 \times 1 \times 1$ mm³, total cuts 28). Visual ratings of white matter hyperintensities (WMH) and counts of DVRS and lacunes were performed by radiologist who were blind to clinical and laboratory details. The degree of WMH was rated visually on axial FLAIR images and was classified according their intensity and location in:

- Periventricular WMH (PWMH): I- No presence or slight cap in ventricles. II-Extense lesions surrounding ventricles.
- Hemispheric WMH (HWMH): I- No presence or difuse lesion. II- Initial confluences or extense confluences.

Dilated Virchow–Robin spaces (> 2 mm) were identified, counted and classified in: None/Few (0–2) and More (≥ 3).

Lacunes were defined as hypointense foci > 3 mm on MPRAGE that were surrounded by white matter or subcortical gray matter and not located in areas with a high prevalence of widened perivascular spaces (e.g., anterior commissure, vertex).

Quantitative maps of the apparent diffusion coefficients (ADC; from DWI, for two b [0 and 1000/s/m²]), related to the water mobility, were calculated as previously detailed [14].

Subclinical brain damage (SBD) was recorded as Yes or No. SBD was considered when brain MRI displayed PWMH/WMHL grade II, and/or DVRS ≥ 3 and/or lacunes.

2.3.1. Atrophy measurements

2.3.1.1. Cortical thickness

For each subject the MRI data set acquired was converted to FreeSurfer data format version 5.3.0. A reliable method was used to calculate cortical thickness in a tessellated model of the cortical surface [15–16]. The technique is composed by several segmentation, normalization, and classification steps. White matter is labeled employing voxel intensity and geometric continuity. The gray/white matter interface is tessellated and has topological defects corrected. Cortical thickness was calculated considering the minimum distance between the interface surface and pial surface. The cortex of each hemisphere was automatically parcellated into 76 regions and the average thickness for each cortical region was calculated. Cortical regions were distributed according to their localization in the cerebral lobes. Aver-

age cortical thickness for each cerebral lobe and overall average cortical thickness were calculated.

2.3.1.2. Linear measures

The following linear measurements were obtained on enlarged images with a built-in distance measurement software: bifrontal index, subarachnoid space, interuncal width, Sylvian fissure, frontal inter-hemispheric fissure width (IHFV), Evan's index, 3rd ventricle [17].

2.4. Ethics

All hypertensive patients signed a written informed consent to participate in the study. The research project was approved by the Institutional Ethics Committee, and all the procedures were in agreement with the principles of the Declaration of Helsinki for studies in human subjects.

2.5. Statistical procedures

Frequencies of categorical variables were calculated and continuous variables with normal distribution were expressed as mean \pm SD. Serum NR2Ab concentration did not follow a normal distribution and was expressed as median and 10–90 percentiles. The χ^2 test was used to evaluate associations between categorical variables and the Mann-Whitney-*U* test to compare medians of continuous data. Spearman's correlation between NR2Ab and measures of brain atrophy were calculated. Bonferroni's correction was applied for multiple testing of correlation coefficients, setting significance at $p < 0.0125$. Multiple regression analysis was carried out to explore the independent associations of clinical, imaging and demographic variables with serum concentrations of NR2Ab. Receiver Operator Characteristics (ROC) curve for NR2Ab as a predictor of subclinical white matter lesions in brain MRI was calculated, based on which sensitivity and specificity were estimated for a specific cutoff value. Statistical calculations were carried out with Statistica8.0 for Windows (StatSoft Inc., 2007), and statistical significance was considered if $p < 0.05$.

3. Results

3.1. Clinical and biomarker studies

The frequency of other pre-existing conditions (PEC) for vascular risk aside from arterial hypertension in the study group is shown in Table 1.

Demographic and clinical characteristics of the subjects included in the study and classified according to the number of PEC in few and more PEC (number of pre-existing conditions present) are presented in Table 2. Gender composition and age were similar in the two groups; nevertheless, BMI was significantly higher and a trend towards more years of HT was observed in the group with more PEC. The distribution according to color of skin was: white-25 (53.2%); mixed-15 (31.9%); black-7 (14.9%), and did not vary with the number of PEC. Frequencies for smoking and family history of CVD did not differ between groups.

Table 1
Frequency of pre-existing conditions (PEC) for vascular risk.

PEC	Subjects (%)
Diabetes mellitus	20 (42.6%)
Hypertensive retinopathy	41 (88.4%)
Dyslipidemia	9 (21.9%)
Overweight/obesity	30 (76.9%)

Table 2
Characteristics of the hypertensive subjects included in the study groups.

Variables	Group I (n = 22) Few PEC	Group II (n = 25) More PEC	Mann-Whitney Z/p
Age	62.0 (44.0–67.0)	62.0 (43.0–71.0)	ns
Female gender	12 (57.1%)	16 (64.0%)	ns
SBP	130 (110–145)	130 (100–158)	ns
DBP	80 (70–90)	80 (70–95)	
Years of HT	10.0 (4–20)	17.5 (5–35)	1.82/0.0660
Body mass index	26.4 (21.7–32.4)	29.6 (25.7–47.2)	2.46/0.0137
Smoking	9 (40.9%)	7 (28.0%)	ns
Family history of CVD	8 (36.4%)	9 (36.0%)	ns

PEC: Pre-existing conditions; CVD: cardiovascular disease.
SBP: systolic blood pressure; DBP: diastolic blood pressure.

The Mann-Whitney test did not reveal a significant difference with respect to serum NR2Ab between HT patients (1.54; range: 1.05–3.38 ng/ml) and the reference group tested in the laboratory (1.97; range: 1.42–2.40 ng/ml) ($Z = -1.21$; $p = 0.2279$). Nevertheless, it should be emphasized that none of the control subjects exhibited NR2Ab > 2.5 ng/ml, while a subgroup of hypertensive patients displayed NR2Ab > 2.5 ng/ml.

Serum levels of NR2Ab in HT patients according to the number of PEC are presented in Fig. 1. Subjects with more PEC displayed higher levels of NR2Ab. However, it should be noted that in the group of HT patients with more PEC, two clusters can be observed: a subgroup of subjects with NR2Ab < 2.0 ng/ml (whose NR2Ab values overlap with those in the group with few PEC), and a subgroup with NR2Ab > 2.0 ng/ml. A proportion comparison analysis between these two subgroups demonstrated that the frequency of diabetes was higher in subjects with NR2Ab > 2.0 ng/ml (81.8%), as compared to those with NR2Ab < 2.0 (57%), while the frequency of the rest of the PEC did not differ.

In HT subjects, NR2Ab levels were not significantly associated with SBP, DBP, years of hypertension, body-mass index, severity of retinopathy or dyslipidemia. However, significantly higher values of

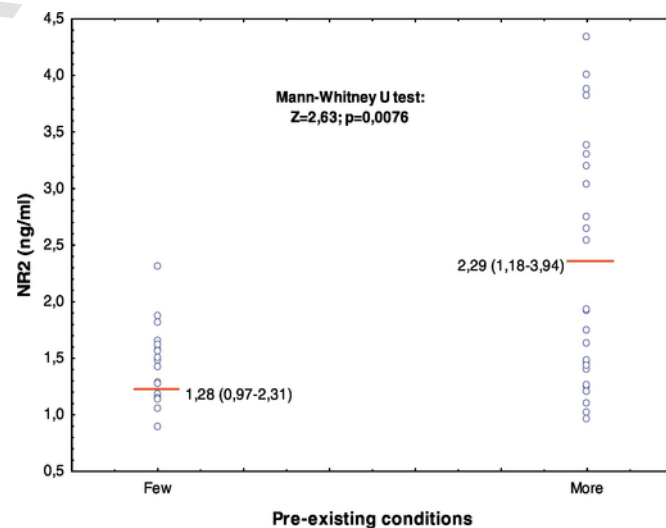


Fig. 1. Levels of NR2Ab in the study groups.

Table 3
Brain MRI findings according to the number of pre-existing conditions (PEC).

MRI Findings	Few PEC (n = 28)	More PEC (n = 19)	χ^2/p
PWMH	I 13 (86.7%)	2 (13.3%)	8.72/0.0032
	II 6 (35.3%)	11 (64.7%)	
SCWMH	I 11 (78.6%)	3 (21.4%)	3.80/0.0512
	II 8 (44.4%)	10 (55.6%)	
DVRS	0-2 13 (81.3%)	3 (18.7%)	6.35/0.0118
	≥ 3 6 (37.5%)	10 (62.5%)	
Lacunes	No 18 (62.1%)	11 (37.9%)	ns
	Yes 1 (33.3%)	2 (66.7%)	
SBD	No 10 (83.3%)	2 (16.7%)	4.57/0.0326
	Yes 9 (45.0%)	11 (55.0%)	

PWMH: periventricular white matter hyperintensities.
 SCWMH: subcortical white matter hyperintensities.
 DVRS: dilated Virchow-Robin spaces.
 SBD: subclinical brain damage.

serum NR2Ab levels were associated with the presence of diabetes mellitus (3.0 ± 1.9 vs 1.57 ± 0.8 ng/l; $t = 3.609$; $p = 0.0008$).

3.2. Neuroimaging and biomarker studies

Brain MRI findings according to the number of PEC are presented in Table 3. Significantly higher frequencies of PWMH, DVRS and SBD and a trend towards more frequent SCWMH were observed in HT subjects with more PEC. Silent lacunes were observed in only 3 patients (2 with More PEC and one with Few PEC). Overall subclinical brain damage was slightly more frequent in patients with more PEC, but it should be noted that the most outstanding result was that most individuals with normal MRI (83.3%) had few PEC.

Evaluating the association between brain MRI findings and serum levels of NR2Ab, we found that more severe PWMH (grade II), DVRS ≥ 3 and SBD (WMH II-DVRS ≥ 3 -Lacunes) were significantly related to higher levels of NR2Ab in HT subjects (Fig. 2). Only three hypertensive patients exhibited lacunar infarcts and no association with NR2Ab was observed.

T1, T2-weighted and FLAIR images showed no cortical lesions in these patients. DWI and ADC imaging were also performed, but they did not offer additional information. No diffusion restriction was observed in DWI.

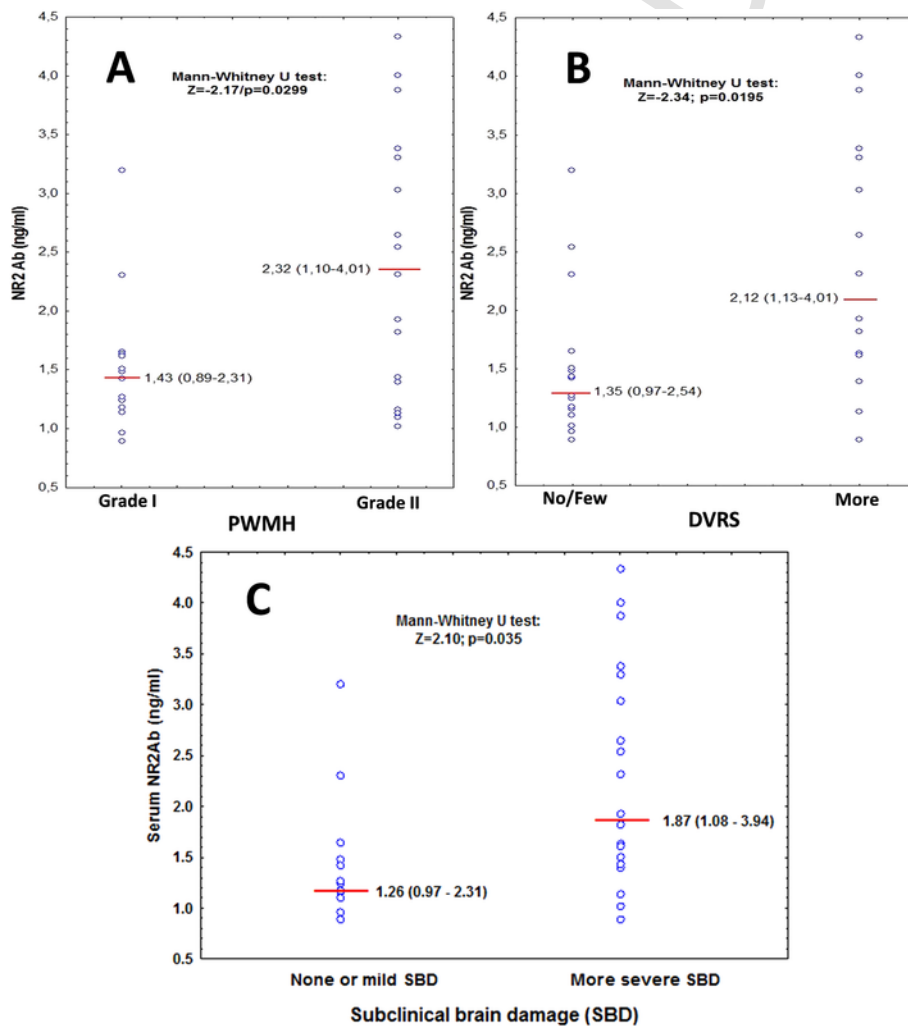


Fig. 2. Levels of NR2Ab according to PWMH (A), DVRS (B) and subclinical brain damage (C) in HT subjects. PWMH: periventricular white matter hyperintensities; DVRS: dilated Virchow Robin spaces.

It caught our attention that in a group of HT patients with more severe brain MRI lesions and especially in those with more severe PWMH and SBD (PWMH II/DVRS ≥ 3 /Lacunes) NR2Ab levels were below 2.0 ng/ml, overlapping with the NR2Ab values of those patients with no lesions or only mild ones (Fig. 2). A proportion comparison analysis demonstrated that the group of subjects with SBD and NR2Ab ≤ 2.0 had significantly lower percentages of PWMH and SCWMH (55.6% and 44.4%) ($p = 0.0198$) as compared to subjects with SBD and NR2Ab > 2.0 (100% and 90.9%) ($p = 0.0459$).

3.2.1. Atrophy measurements

Measurements for cortical thickness, linear measures and correlation with NR2Ab concentration in HT subjects are shown in Table 4. A strong association between frontal cortical thickness and frontal IHFW ($r = -0.65$; $p = 0.0044$) was observed, but no other association was found between regional and overall cortical thickness and linear measures. Spearman's correlation coefficients showed no association between NR2Ab levels and measurements of cortical thickness. Although a trend towards a positive correlation between frontal cortical thickness and NR2Ab concentration was observed, Bonferroni's correction rendered it as non-significant. On the other hand, a strong inverse correlation was observed between NR2Ab levels and frontal interhemispheric fissure width (IHFW) (Fig. 3). No association was observed with the other linear measures of atrophy: bifrontal index, subarachnoid space, interuncal width, Sylvian fissure, Evans' index and 3rd ventricle.

HT subjects with more PEC and NR2Ab ≤ 2.0 ng/ml had larger frontal subarachnoid space and interhemispheric fissure width than subjects with more PEC and NR2Ab > 2.0 ng/ml (medians: 12.5 mm vs 7.1 mm, and 11.0 mm vs 8.6 mm, respectively), these differences were statistically significant for both variables: frontal subarachnoid space ($Z = -2.36$; $p = 0.0185$) and IHFW ($Z = -2.28$; $p = 0.022$). Very similar results were obtained when atrophy measurements were

Table 4
Brain atrophy measurements and correlation with serum NR2Ab concentration in HT subjects.

Brain atrophy measurements			Spearman correlation vs NR2Ab (r/p)
Cortical thickness (mm)	Frontal	2.55 (2.47–2.69)	0.47/0.030
	Occipital	2.16 (2.01–2.30)	0.17/0.454
	Parietal	2.26 (2.16–2.39)	0.24/0.285
	Temporal	2.70 (2.52–2.90)	0.23/0.286
	Overall	2.39 (2.32–2.56)	0.35/0.115
Linear measures (mm)	Bifrontal index	0.318 (0.27–0.36)	0.05/0.810
	Subarachnoid space	10.0 (5.0–15.0)	-0.16/0.455
	Interuncal distance	23.0 (18–28.1)	-0.03/0.902
	Sylvian fissure	8.1 (3.6–12.6)	-0.17/0.428
	Interhemispheric fissure	10.0 (5.0–13.0)	-0.666/0.0004*
	Evans' index	0.27 (0.23–0.29)	0.180/0.378
3rd ventricle	6.0 (3.0–9.1)	-0.11/0.606	

Median (10–90th percentiles).

* Significant after Bonferroni correction for Spearman's correlation.

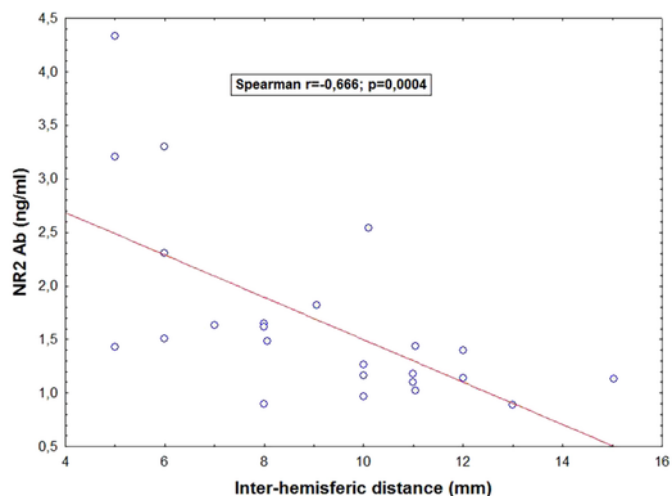


Fig. 3. Correlation between NR2Ab levels and frontal interhemispheric fissure width.

evaluated in HT subjects with more severe brain MRI lesions and NR2Ab < 2.0 (data not shown).

3.3. Multivariate regression analysis and Receiver Operator Characteristics (ROC) curve

Multivariate regression analysis was conducted with NR2Ab as the dependent variable, and age, PEC and brain MRI findings displaying significant associations with NR2Ab in the univariate analysis, as independent variables (PWMH, DVRS and IHFW) (Table 5). The results of the multivariate analysis showed that IHFW and PWMH were the only variables independently associated with serum NR2Ab concentration. No interactions between the categorical variables in the model were observed.

ROC analysis was conducted to evaluate if serum NR2Ab levels could predict subclinical brain damage in HT patients. The area under the curve (AUC) showed that for a cutoff level of 1.7 ng/ml, serum NR2Ab predicted PWMH with a sensitivity and specificity of 65% and 87% respectively (Fig. 4). The sensitivity and specificity of NR2Ab for predicting the other asymptomatic brain lesions (HWMH, DVRS and SBD) were similar.

4. Discussion

The present work evaluated the usefulness of employing serum NR2Ab levels for the prediction of subclinical brain damage in hypertensive patients and its relation with PEC for vascular risk in these

Table 5
Multivariate regression analyses with serum NR2Ab as the dependent variable.

Dependent variable: NR2Ab (multiple $r = 0.875$; $F = 4.72$; $p = 0.006$)			
Independent variables	t	p	Beta (95% CI)
Intercept	5.07	0.002	–
Age	-1.044	0.315	-0.177 (-0.544 to 0.189)
PEC	-0.878	0.396	-0.153 (-0.529 to 0.223)
IHFW	-3.565	0.003	-0.667 (-1.071 to -0.263)
PWMH	2.261	0.041	0.400 (0.781 to 0.018)
DVRS	-1.445	0.172	-0.233 (-0.582 to 0.115)

PEC: pre-existing conditions.

IHFW: frontal interhemispheric fissure width.

PWMH: periventricular white matter hyperintensities.

DVRS: dilated Virchow Robin spaces.

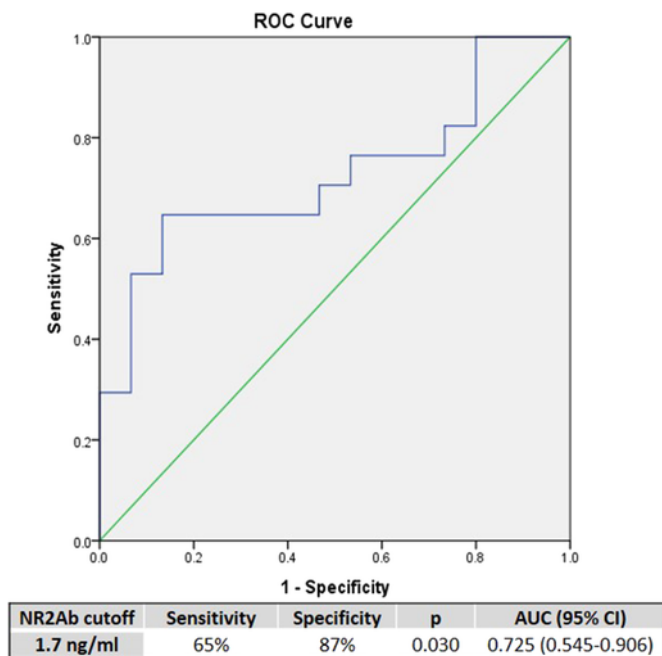


Fig. 4. Receiver Operator Curve (ROC) for the prediction of PWMH by serum NR2Ab.

subjects (hypertensive retinopathy, overweight/obesity, diabetes mellitus and dyslipidemia). Higher NR2Ab levels in hypertensive patients were found to be related with more PEC and with more severe brain MRI changes, while a linear indicator of frontal cortical atrophy - width of the interhemispheric fissure [17] was inversely related.

PEC for vascular risk are known to accelerate the atherosclerotic process in the different vascular beds - including the brain - through endothelial dysfunction and low grade inflammation, subsequently leading to chronic hypoperfusion and cerebral small vessel disease [2,18–19]. Overall, brain injury involves neurotoxic mechanisms, impaired permeability of the blood-brain barrier, and immune response, which result in activation of intra and extracellular proteases. Brain-derived proteins and peptides from the central nervous system can thus be proteolytically degraded in the interstitial space and released into the bloodstream. In chronic conditions, NR2 peptides, a subunit of NMDA membrane receptor, induce the production of autoantibodies (NR2Ab) in the blood [8,20]. This situation could explain why raised levels of NR2Ab were found in subjects with more PEC and more severe MRI changes (PWMH, dilated Virchow-Robin spaces and/or lacunes) in this work.

NR2Ab levels were not related to one specific PEC, but depended more on the confluence of several comorbidities, although diabetes mellitus seemed to contribute with a more important weight. This is supported by the fact that the subset of subjects with NR2Ab ≤ 2.0 ng/ml in the group with more PEC have significantly lower frequencies of hypertension, diabetes and dyslipidemia than those with NR2Ab > 2.0 ng/ml. Brain damage caused primarily by HT² is enhanced by the sum of vascular PEC, especially diabetes. The association of DM with NR2Ab levels could be associated to additional damage at the microvascular level caused by hyperglycemia, including low perfusion rates, thickening of capillary walls and abnormal proliferation of endothelial cells with increased vascular permeability [21–22]. This has been reported to result in loss of white matter integrity and altered functional connectivity [23–24]. Several studies have reported an association between DM and WMLs [25–26]. Very recently an increased prevalence of lesions indicative of cerebral

small vessel disease (WML and/or lacunes) was reported in patients with diabetic retinopathy [27].

Although more severe brain MRI findings denoting cerebral small vessel disease were associated with higher NR2Ab levels (PWMH grade II, DVRS ≥ 3 and SBD); brain atrophy related variables displayed no correlation with NR2Ab, except for frontal interhemispheric fissure width, which revealed a highly significant inverse correlation with NR2Ab, while frontal cortical thickness displayed a trend to correlate positively. The subsequent multivariate analyses indicated that the main MRI variables independently associated with serum NR2Ab levels were PWMH and frontal interhemispheric fissure width; while age, presence of PEC and of DVRS did not contribute. This poses a somehow complicated situation, because on one side these brain lesions could be related to increased NMDA neuroreceptor cleavage under chronic ischemic conditions, as previously described [10], with an increase of NR2Ab as a neurochemical expression of damage to the brain tissue. On the other hand, frontal cortical atrophy could indicate decreased cortical tissue volume, which could imply that there are less NMDA receptors to be damaged and released. Although our study could not statistically confirm an association between frontal cortical thickness and NR2Ab levels, a trend towards a positive correlation was observed, while for the other cerebral lobes this was not so. The strong inverse association we found between frontal cortical thickness and frontal interhemispheric fissure width supports that both variables indicate frontal atrophy as expected. Possibly the poorer association of frontal cortical thickness with NR2Ab is because the width of the interhemispheric fissure points to overall frontal lobe atrophy, where not only cortex is involved, but also white matter.

It is well known that NMDA receptors have a widespread distribution in the brain, with their highest expression in the cerebral cortex and lowest in the pons-medulla [28–29]. Nevertheless, very recently Christensen et al. reported that NMDA receptors co-localize with myelin, and oligodendroglial cell bodies and processes, indicating that they are also expressed in human white matter [30]. This could explain why the interhemispheric fissure width correlates so well with NR2Ab in this study.

If NMDA receptors are localized throughout the cortex, why is it that only frontal atrophy displays an inverse correlation with circulating NR2Ab levels? A study describing the regional distribution of the NMDA receptor subunits NR2A, 2B, 2C and 2D in rat brain reported that NR2A immunoreactivity was found in almost all regions of the brain, whereas NR2B staining was restricted to forebrain [31]. On the other hand, the aging process has been characterized by atrophic changes normally affecting the frontal lobes first, followed by the parietal lobes with consecutive enlargement of the lateral ventricles [32]. These findings could partly support the inverse correlation found in our study between serum NR2Ab levels and frontal cortical atrophy, as our patients could be submitted to an early stage of accelerated brain aging related to the risk factors they convey, favoring cerebral small vessel disease [33–34].

Previous studies have shown that increasing small vessel disease is associated with increases in WMH volume and brain atrophy [35–36]. However, the direction of this relation is still controversial [37]. A recent paper demonstrated that the rate of WMH growth is strongly correlated with regional gray matter atrophy, and this atrophy contributes most to the secondary reductions in global brain volume [33].

Thus, our results indicate that greater frontal atrophy and more severe PWMH appear to work in opposite directions with respect to NR2Ab levels. This possibly explains why there are subgroups of patients that have more PEC and more severe brain lesions, while serum NR2Ab levels are within the normal range. This could be a lim-

itation in its usefulness for the prediction of subclinical brain damage, especially when the patient concomitantly has frontal lobe atrophy.

Serum NR2Ab exhibited a sensitivity of 65% and a specificity of 87% for the prediction of asymptomatic PWMH at a cut off level of 1.7 ng/l. The sensitivity is lower than the one we reported previously for NSE (80%) in hypertensive patients as a predictor of subclinical brain damage, but the specificity markedly increased with respect to that reported for NSE (58%) [7]. Lower sensitivity could be due to two main causes: 1) the brain atrophy issue we discussed above and 2) unreported chronic inflammatory or autoimmune disorders, because NMDA receptors are also located on the surface of epithelia of microvessels that form the blood-brain barrier [38] and could respond to inflammation without brain damage. Nevertheless, a high specificity is suitable when our main goal is to filter those cases that are at a lower risk to develop cerebrovascular disease in the community, thus focusing the physician's attention on those cases with higher NR2Ab levels.

There are some limitations in our study. First, the investigation was conceived as a pilot study and the sample was obtained from a single health area in Havana; second, the sample number is small, thus reducing the strength of the statistical associations between NR2Ab levels and the MRI findings related to white matter lesions and brain atrophy; third, to our knowledge the kinetics of the release of NR2 peptides to the bloodstream during the course of asymptomatic cerebral small vessel disease has not been investigated, thus the NR2 antibody response may not necessarily be stable or linear over time with disease progression.

Concluding, higher serum NR2Ab levels are related with more severe brain MRI lesions denoting cerebral small vessel disease, but the presence of frontal lobe atrophy acts in the opposite direction, lowering NR2Ab possibly due to a decrease in tissue volume. Thus, screening for early brain damage with NR2Ab would probably be more useful in younger populations at risk, where age-related cortical atrophy has not yet fully established. These preliminary results need future confirmation, but they support our previous thoughts on the utility of investigating circulating brain specific proteins and/or autoantibodies directed against them for the early detection of asymptomatic brain damage in high risk populations [7]. This could contribute information for undertaking the clinical management of these "high risk" subjects more aggressively in order to reduce future vascular complications and upgrade their personal cardiovascular risk.

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