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Elevated circulating metalloproteinase 7 predicts recurrent cardiovascular events in patients with carotid stenosis: a prospective cohort study

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Abstract

Background: Major adverse cardiovascular events are the main cause of morbidity and mortality over the long term in patients undergoing carotid endarterectomy. There are few reports assessing the prognostic value of markers of inflammation in relation to the risk of cardiovascular disease after carotid endarterectomy. Here, we aimed to determine whether matrix metalloproteinases (MMP-1, MMP-2, MMP-7, MMP-9 and MMP-10), tissue inhibitor of MMPs (TIMP-1) and in vivo inflammation studied by ¹⁸F-FDG-PET/CT predict recurrent cardiovascular events in patients with carotid stenosis who underwent endarterectomy.

Methods: This prospective cohort study was carried out on 31 consecutive patients with symptomatic (23/31) or asymptomatic (8/31) severe (> 70%) carotid stenosis who were scheduled for carotid endarterectomy between July 2013 and March 2016. In addition, 26 healthy controls were included in the study. Plasma and serum samples were collected 2 days prior to surgery and tested for MMP-1, MMP-2, MMP-7, MMP-9, MMP-10, TIMP-1, high-density lipoprotein, low-density lipoprotein, high-sensitivity C-reactive protein and erythrocyte sedimentation rate. ¹⁸F-FDG-PET/CT focusing on several territories' vascular wall metabolism was performed on 29 of the patients because of no presurgical availability in 2 symptomatic patients. Histological and immunohistochemical studies were performed with antibodies targeting MMP-10, MMP-9, TIMP-1 and CD68.

Results: The patients with carotid stenosis had significantly more circulating MMP-1, MMP-7 and MMP-10 than the healthy controls. Intraplaque TIMP-1 was correlated with its plasma level ($r = 0.42$ $P = .02$) and with ¹⁸F-FDG uptake ($r = 0.38$ $P = .05$). We did not find any correlation between circulating MMPs and in vivo carotid plaque metabolism assessed by ¹⁸F-FDG-PET. After a median follow-up of 1077 days, 4 cerebrovascular, 7 cardiovascular and 11 peripheral vascular events requiring hospitalization were registered. Circulating MMP-7 was capable of predicting events over and above the traditional risk factors (HR = 1.15 $P = .006$). When the model was associated with the variables of interest, the risk predicted by ¹⁸F-FDG-PET was not significant.

Conclusions: Circulating MMP-7 may represent a novel marker for recurrent cardiovascular events in patients with moderate to severe carotid stenosis. MMP-7 may reflect the atherosclerotic burden but not plaque inflammation in this specific vascular territory.

Keywords: Biomarker, Carotid endarterectomy, Matrix metalloproteinases, Positron emission tomography

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Background

Atherosclerotic disease of the carotid artery is responsible for 30% of ischemic strokes [1]. Indeed, patients undergoing carotid endarterectomy have been shown to be at risk of major adverse cardiovascular events [2]. History of peripheral artery disease [2], chronic renal insufficiency [3] and ischaemic heart disease [4] have been identified as predictors of cardiovascular events following endarterectomy.

Inflammation and proteolytic activity are strongly associated with plaque instability and rupture and may predict the risk of cardiovascular events [5, 6]. However, there are few reports assessing the prognostic value of markers of inflammation in relation to the risk of cardiovascular disease after carotid endarterectomy [7].

Defects in the synthesis and breakdown of the extracellular matrix are considered key factors in the development of atherosclerotic disease and its thrombotic complications, and metalloproteinases (MMPs) are important mediators of these processes [8]. The relevance of MMPs in atherosclerosis has raised the possibility that they may be used as cardiovascular biomarkers [9]. Several studies have shown that different MMPs are more prominent in the blood of patients with carotid atherosclerosis or ischaemic stroke [7, 10–13]. Higher circulating levels of MMP-2, MMP-9, MMP-7, MMP-12, and TIMP-1 (tissue inhibitor of matrix metalloproteinase 1) might contribute to carotid plaque instability due to inflammatory mechanisms [7, 10]. However, studies into the relationship between MMPs and the metabolic activity of the vascular wall, measured by ¹⁸fluorine-fluoro-deoxyglucose-positron emission tomography combined with computed tomography (¹⁸F-FDG-PET/CT), as an indirect measure of carotid inflammation *in vivo*, have produced conflicting results [11, 12, 14].

Here, we aimed to determine whether matrix metalloproteinases (MMP-1, MMP-2, MMP-7, MMP-9 and MMP-10), tissue inhibitor of MMPs (TIMP-1) and vascular ¹⁸F-FDG-PET/CT predict recurrent cardiovascular events in patients with carotid stenosis who underwent endarterectomy.

Methods

Study population

In this prospective, longitudinal, hospital-based cohort study, 31 consecutive patients with symptomatic or asymptomatic severe (>70%) carotid stenosis who were scheduled for carotid endarterectomy at Clínica Universidad de Navarra or Hospital de Navarra (Spain) were recruited between July 2013 and March 2016. No patient was included postoperatively, and patients were excluded if there was evidence of active infection, generalized or local inflammatory disease (discounting atherosclerosis), chronic kidney disease, or neoplastic disease or if they were on haemodialysis. A complete medical history was

recorded for all patients, including details of previous stroke, transient ischaemic attack, coronary disease (myocardial infarction, angina or revascularization), intermittent claudication, arterial hypertension, smoking status, diabetes mellitus, body mass index, and grade of bilateral carotid stenosis and medication. Patients were selected for endarterectomy based on consensus among the multidisciplinary team members of the Carotid Clinic, which included vascular surgeons, cardiologists, and neurologists, who evaluated the patients before surgery. All procedures were performed under general anaesthesia by the vascular surgeons who have extensive experience in carotid endarterectomy. A blood analysis was performed no more than 2 days prior to surgery. Active smokers or patients who had stopped smoking within 2 years of recruitment were considered smokers. A carotid ultrasonography assessment was performed to determine the grade of stenosis, according to the latest guidelines [15], and this was confirmed by magnetic resonance angiography with coronal fast 3D T1-weighted sequences after administration of a single dose of intravenous gadolinium contrast, with digital subtraction and maximum-intensity projection reconstructions, using a 3-T MRI scanner (Magnetom Trio Tim, Siemens, Erlanger, Germany). Stenosis was measured following NASCET recommendations [16]. The patients were classified as symptomatic if they had had a stroke, transient ischemic attack (TIA) or *amaurosis fugax* ipsilateral to the stenotic carotid artery. Carotid ultrasonography in these patients was performed as part of routine clinical practice in the stroke unit. Asymptomatic carotid stenosis was detected in patients with a high cardiovascular risk undergoing carotid ultrasonography for screening. History of previous stroke in asymptomatic patients included non-atherosclerotic stroke. One “asymptomatic patient” was diagnosed with cancer following ¹⁸F-FDG-PET/CT imaging at the beginning of the study and was therefore excluded from the cohort. In addition, from a cohort of healthy individuals free from cardiovascular diseases, workers at the University of Navarra (Spain) who attended the Internal Medicine outpatient clinic for a general check-up, 26 subjects age and sex-matched were included (mean age 66.8 ± 3.0, males/females 16/10) for measurement of blood MMP-1, MMP-2, MMP-7, MMP-9, MMP-10 and TIMP-1. All protocols were approved by the Committee for Medical and Research Ethics. Participants gave written informed consent. Samples from patients included in the study were provided by the Biobank of the University of Navarra and were processed following standard operating procedures approved by the Research Ethics and Scientific Committees.

Biomarker assessment

Blood samples were collected after overnight fasting, no more than 2 days prior to carotid endarterectomy. The

blood was allowed to clot and was centrifuged at 2000 \times *g* for 10 min using a refrigerated centrifuge. The serum was removed, aliquoted and stored at -80°C until it was assayed. Plasma was obtained from citrated blood by centrifugation at 2000 \times *g* for 10 min using a refrigerated centrifuge, aliquoted and stored at -80°C until assayed. MMP-1, MMP-2, MMP-7, MMP-9 and MMP-10 were measured in citrated plasma with a bead-based multiplex assay using Luminex technology (MILLIPLEX MAP Human MMP Magnetic Bead Panel 2, ref. HMMP2MAG-55 K; Merck, Darmstadt, Germany). TIMP-1 was assayed in serum using a specific enzyme-linked immunosorbent assay (Human TIMP-1 Quantikine ELISA Kit, ref. DTM100; R&D Systems, Minneapolis, USA). MMPs and TIMP-1 were measured following the manufacturers' instructions. The inter- and intra-assay coefficients of variation for these ELISAs were $< 6\%$.

Total serum cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides were measured in fasting blood samples using standard laboratory techniques. Low-density lipoprotein (LDL) cholesterol was estimated using the Friedewald equation, and high-sensitivity C-reactive protein (hs-CRP) was measured in an immunoassay (Immulite; Diagnostic Products Corporation, Los Angeles, CA, USA). The erythrocyte sedimentation rate (ESR) was also analysed by the Westergren method [17].

Histological assessment

Vascular segments were removed carefully at endarterectomy to preserve plaque structure. Specimens from the 31 patients undergoing carotid endarterectomy were cut into transverse pieces and fixed immediately in 4% paraformaldehyde, decalcified for 24 to 72 h at room temperature (Osteosoft; Merck Millipore, Darmstadt, Germany) and embedded in paraffin. Haematoxylin-eosin and van Gieson staining were used to examine the microscopic morphological features of the tissue (Nikon Optiphot-2, Japan).

For protein immunolocalization, serial tissue sections from all patients were analysed by immunohistochemistry. Antigen retrieval was performed using 10 mM citrate, pH 6.0, (MMP-10) or TE, pH 9.0, (MMP-9, TIMP-1, CD68) and a DakoCytomation Pascal pressure chamber (Dako) at 95°C for 30 min. Slides were treated with H_2O_2 for 10 min to block endogenous peroxidase activity, and sections were blocked with 5% normal goat serum in PBS. Tissue sections were incubated with antibodies targeting MMP-10 (rabbit polyclonal, Acris ref. AP07210PU-N, dil. 1:20), MMP-9 (rabbit polyclonal, Thermo Fisher ref. PA5-13199, dil. 1:800), TIMP-1 (mouse monoclonal, Merck ref. MAB13429, dil. 1:400) and CD68 (mouse monoclonal, Dako ref. GA609, dil. 1:2000), as described previously [18]. After washing, sections were incubated for 30 min with

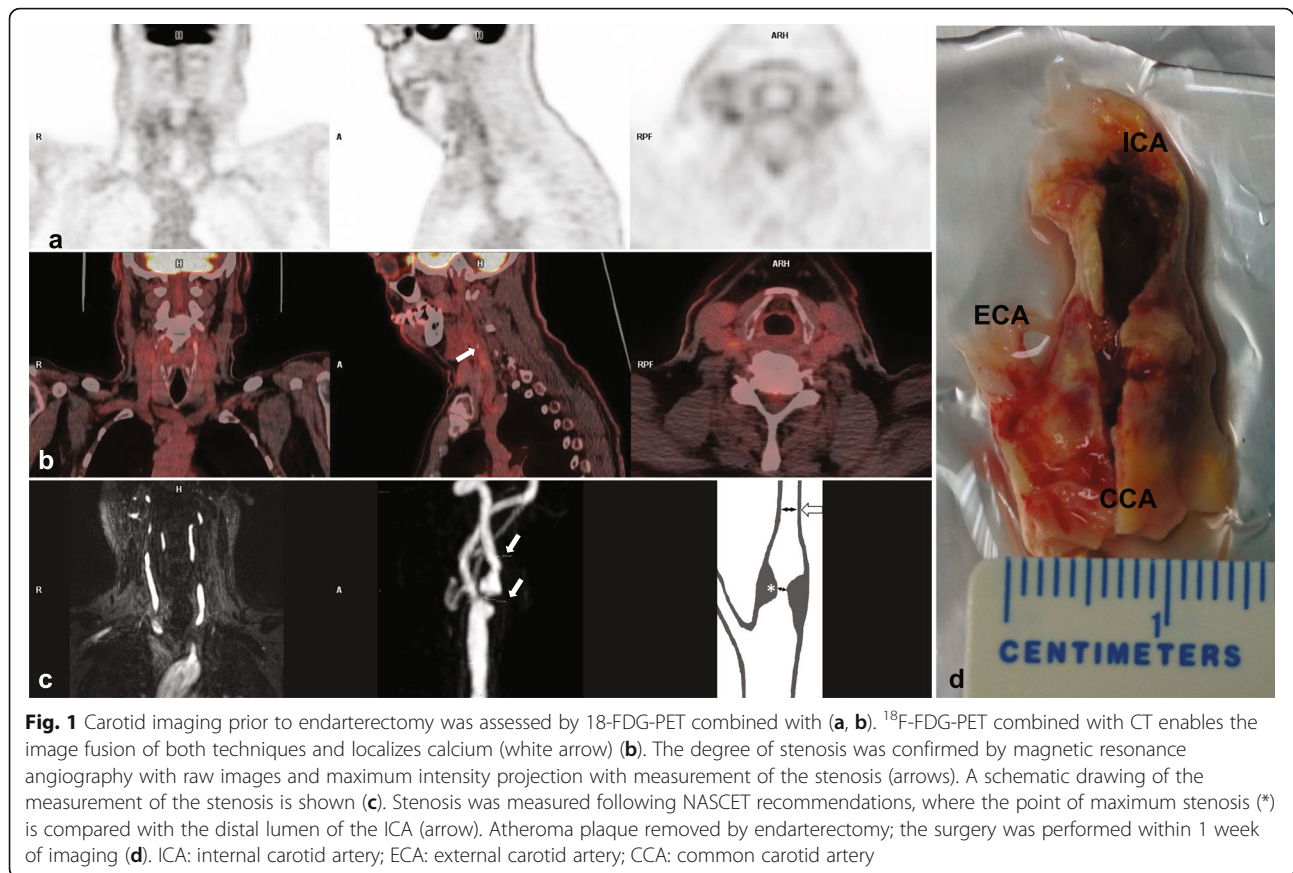
HRP-labelled polymer conjugated to anti-rabbit (ref. K4003) or anti-mouse (ref. 4001) secondary antibodies (Dako). The signal was revealed by using the DAB+ Substrate Chromogen System (Dako ref. K3468). Negative controls, prepared by omitting the primary antibody, were included in each processed batch. Three independent observers, blinded to clinical data, used a semi-quantitative visual grading scale (Additional file 2) to assess the immunostaining (0–5 scale).

Vascular ^{18}F -FDG PET/CT imaging

^{18}F -FDG-PET/CT imaging that focused on carotid wall metabolism was performed in 29 of the 31 patients (Fig. 1), as there was no presurgical availability in 2 symptomatic patients. The maximum standardized uptake value (SUVmax) was used to measure the ^{18}F -FDG uptake at the ipsilateral and contralateral carotid arteries. The target-to-background ratio (TBR) was calculated as the ratio of SUVmax measured in the atherosclerotic plaque or the arterial vessel to the venous blood pool SUVmax to correct for blood-pool uptake. Venous activity was measured as mean SUV in the ipsilateral jugular veins for carotid studies, and inferior vena cava for the other territories (aortic arch and the abdominal aorta).

Patient follow-up

Patients were followed at the outpatient service of the Department of Neurology and/or Vascular Surgery every 3 or 6 months and at the Emergency Department (median follow-up 1077 days [interquartile range, 689–1484 days]) until May 2018. The primary outcome of the study was defined as the composite of cardiovascular death, a nonfatal cardiovascular event (including stroke and myocardial infarction) or rehospitalization for acute ischaemia (TIA, *amaurosis fugax*, *angina pectoris* and peripheral artery disease) as previously described [19]. These outcomes were determined through a review of the patient's medical history. Peripheral artery disease was diagnosed with the combination of compatible symptoms, physical examination, ankle brachial index and duplex ultrasound [20]. Additional imaging (with CT angiography, MR angiography, or digital subtraction angiography) was performed when revascularization was considered [21]. Patients with symptoms of coronary disease were reviewed by the cardiology team, and coronary angiography or other complementary tests were performed in accordance with the latest guidelines [22]. The period between surgery and the day of outcome was calculated, and the overall survival and presence of recurrent cardiovascular events were calculated from the day of surgery to the date of the last follow-up available. No patients were lost to follow-up.



Statistical methods

All categorical variables are expressed as frequencies (ratios), and all the continuous variables are expressed as the mean (\pm standard error). Statistical analysis included a Shapiro-Wilk test for normality. Pearson's correlation coefficient and Spearman's correlation coefficient were used to test the correlations between MMPs and SUVmax or TBR. A Bonferroni correction for multiple comparisons (using as a reference for the p -value 0.05 divided by the number of comparisons) was made. An unpaired Student's t test was used to compare the MMP values between the patients (symptomatic and asymptomatic combined) and healthy controls, and a Cox regression for recurrent events with multiple imputation for missing data was used to determine the role of MMPs in event prediction. Hazard ratios were obtained after adjusting for relevant covariates. Receiver operating characteristic (ROC) curves were used to analyse the ability of MMP-7 to predict events in the population of subjects after fitting a logistic regression model. Risk factors, including age, sex, tobacco use, cholesterol levels, hypertension and diabetes mellitus, were grouped following the recommendations of a previous study [23]. Considering that the population of Navarra has higher levels of HDL-cholesterol on average, a specifically adapted version of the Framingham-Wilson Coronary Risk Equation was used to

assess global cardiovascular risk: Risk score for CORonary events in the population of NAVarra (RICORNA) [24].

Statistical significance was established at $p < .05$, and the statistical analysis, including sample size computations, was performed with the Stata software package version 15.1 for Mac (StataCorp; College Station, TX, USA).

The size of the available sample is rather small, which was the reason some non-parametric analysis was performed. For a Cox regression analysis, this sample size jointly with a significance level of 0.05 and a test power of 0.20 allows the detection of hazard ratios of size less than 0.0001 assuming a standard deviation of 0.04, which is quite good since for that we have obtained a hazard ratio of 0.006. For the worst standard deviation (we have 2.7), a hazard ratio of size 0.8300 could be detected, which is reasonably good since we obtained a hazard ratio of 1.7.

Results

This study cohort was composed of 31 subjects (mean age \pm standard deviation 67.4 ± 2.6 years, 21/31 men) after the exclusion of one patient due to the diagnosis of bladder cancer following the ¹⁸F-FDG-PET/CT study. A similar population of 26 healthy controls (mean age

66.8 ± 3.0 years, 16/26 men) was also analysed. Among the patients, 23/31 were symptomatic (see “Study population” above), and 8/31 were asymptomatic (see Table 1 for the patients’ characteristics). The rates of hypertension and diabetes mellitus were similar, whereas other risk factors differed between the two groups (Table 1). When the baseline plasma levels of MMPs and TIMP-1 were compared, significantly higher levels of MMP-1, MMP-7 and MMP-10 were evident in the patient group, while no such differences were found for MMP-2, MMP-9 or TIMP-1 (Table 2).

MMPs and ¹⁸F-FDG PET/CT imaging

No significant correlation was found between blood levels of MMPs and the SUV of the symptomatic carotid artery (for MMP-7, $r = -0.20$ $p = .28$) or the contralateral carotid artery (for MMP-7, $r = -0.23$ $p = .21$). Similar results were obtained with the normalized TBR for the ipsilateral carotid artery (for MMP-7, $r = -0.35$ $p = .06$) or for the contralateral carotid artery (for MMP-7, $r = -0.24$ $p = .21$). SUVmax values were consistently correlated between neighbouring territories (carotid and aortic arch,

Table 2 Baseline plasma levels of MMPs and TIMP-1

Biomarker	Patients (n = 31)	Controls (n = 26)	
MMP-1, ng/ml	11.8 (9.5)	6.2 (4.1)	$p = .003$
MMP-2, ng/ml	125.3 (6.5)	135 (6.4)	$p = .3$
MMP-7, ng/ml	20.4 (6.4)	17.5 (4.1)	$p = .02$
MMP-9, ng/ml	117.8 (10.7)	123.5 (12.4)	$p = .7$
MMP-10, pg/ml	726.7 (81.6)	513.2 (40.0)	$p = .01$
TIMP-1, ng/ml	211.6 (9.4)	195.5 (8.1)	$p = .2$

Data are expressed as the mean (standard deviation). *MMP* metalloproteinase, *TIMP* tissue inhibitor of matrix metalloproteinases

$r = 0.41$ $p = .03$; carotid and abdominal aorta, $r = 0.51$ $p = .02$). However, none of the blood levels of MMPs was correlated with ¹⁸F-FDG uptake in the carotid territory, aortic arch or abdominal aorta (see table IIS in the supplementary material).

MMPs and histological findings

All endarterectomy samples corresponded to advanced atherosclerotic plaques (types V–VIII) according to the American Heart Association (AHA) histological classification [25].

Table 1 Patients’ characteristics

Clinical history and characteristics	Symptomatic (n = 23)	Asymptomatic (n = 8)		Controls (n = 26)	
Age, years (SD)	67.3 (16.0)	66.3 (22.5)	$p = .4$	66.8 (3.0)	$P = .4$
Male (%)	15 (65)	6 (75)	$p = .3$	16 (62)	$P = .3$
Hypertension (%)	17 (74)	6 (75)	$p = .6$	0	
Diabetes mellitus (%)	8 (35)	2 (10)	$p = .6$	0	
Prediabetes (%)	1 (4)	2 (25)	$p = .12$	0	
Coronary disease (%)	7 (30)	1 (12)	$p = .4$	0	
Tobacco (%)	12 (52)	7 (87)	$p = .1$	0	
Previous stroke/TIA (%)	4 (17)	2 (25)	$p = .5$	0	
BMI, kg/m ² (SD)	27.5 (1.1)	29.2 (1.3)	$p = .7$	N/A	
Grade of ipsilateral stenosis % (SD)	82.6 (16.2)	85.0 (14.2)	$p = .6$	N/A	
Grade of contralateral stenosis % (SD)	37.4 (25.0)	35.0 (23.2)	$p = .7$	N/A	
Medication					
Statins (%)	13 (57)	7 (87)	$p = .4$	0	
Anti-aggregants (%)	11 (48)	7 (87)	$p = .04$	0	
Anti-coagulants (%)	0	1 (12)	$p = .02$	0	
Blood analysis					
Leukocytes, 10 ⁹ /l (SD)	7.69 (0.43)	7.50 (0.73)	$p = .4$	N/A	
Platelets, 10 ⁹ /l (SD)	210.3 (10.1)	210.7 (13.5)	$p = .5$	N/A	
ESR, mm/h (SD)	10.86 (1.30)	4.83 (1.25)	$p = .03$	N/A	
hs-CRP, mg/l (SD)	1.94 (0.41)	1.38 (0.74)	$p = .3$	N/A	
LDL cholesterol, mg/dl (SD)	102.3 (6.2)	80.0 (10.0)	$p = .04$	N/A	
HDL cholesterol, mg/dl (SD)	44.3 (2.4)	49.7 (5.8)	$p = .8$	N/A	
Triglycerides, mg/dl (SD)	119.4 (14.5)	134.6 (27.4)	$p = .6$	N/A	

SD standard deviation, TIA transient ischemic attack, BMI body mass index, N/A not applicable, ESR erythrocyte sedimentation rate, hs-CRP high-sensitivity C-reactive protein, LDL low-density lipoprotein, HDL high-density lipoprotein, unk unknown, N/A: not applicable. Data are expressed as the total number (percentage) or mean (standard deviation)

The median of the semi-quantitative visual grading scale (0–5) for each MMP immunostaining is available in Table IS in the supplementary material. There was a significant association between CD68 and MMP-9 ($r_s = 0.42$, $p = 0.009$) or TIMP-1 ($r_s = 0.38$, $p = .018$), even after Bonferroni correction, but not with MMP-10 ($r_s = 0.28$). A moderate correlation was found between plasma and intraplaque TIMP-1 values ($r = 0.42$; $p = .02$), but there was no association between intraplaque MMP-9 or MMP-10 and their plasma concentration. The correlations between plasma MMPs and histological findings are shown in Table 3. Intraplaque TIMP-1 was correlated with its plasma level ($r = 0.42$ $p = .02$) and with ^{18}F -FDG uptake ($r = 0.38$ $p = .05$). A weak correlation did appear to exist between intraplaque CD68 (macrophage marker) and ^{18}F -FDG-PET ($r = 0.31$), yet this was not statistically significant ($p = .12$). No correlation was found between intraplaque MMPs, TIMP-1, CD68 and hs-CRP or ESR.

MMPs as predictors of recurrent cardiovascular events

After a mean follow-up of 1004 days, 4 cerebrovascular (2 ischemic strokes and 2 transient ischaemic attacks), 7 cardiovascular (4 myocardial infarctions, 3 *angina pectoris*) and 11 peripheral vascular events requiring hospitalization were registered. No deaths were recorded during the follow-up. As expected, the RICORNA risk score predicted future events (HR = 1.08 $p = .002$). When other variables were added to the model, including MMPs and the results of ^{18}F -FDG-PET, circulating MMP-7 was the only MMP capable of improving the predictive value of RICORNA (HR = 1.15 $p = .006$). The TBR of the contralateral carotid artery alone seemed to be a good predictor of such events (HR = 3.5 $p = .05$), although when the model was associated with the variables of interest, namely, RICORNA and MMP-7, the risk was not significant for TBR (HR = 2.5 $p = .15$). The inclusion of other variables, such as the remaining MMPs, TIMP-1, CRP or ESR, and ^{18}F -FDG-PET, failed to improve the prediction of cardiovascular events. Twenty multiple imputation for the 8 missing values in the variable MMP-7 and ^{18}F -FDG-PET were applied. When using only RICORNA, the model was

predictive ($P = 0.001$) and when using MMP-7 and ^{18}F -FDG-PET the model was also predictive ($P = 0.019$). This proves the good predictive behaviour of MMP-7 thanks to the use of multiple imputation for missing data. MMP-7 levels were not significantly different in patients with and without coronary disease. When ROC curves were studied with a logistic regression for MMP-7, a cut-off point of 20.48 ng/ml was established (for a probability of .4986), which led to a sensitivity of 61%, a specificity of 87%, and a positive predictive value of 80%. This probability came from maximizing the ROC (Fig. 2).

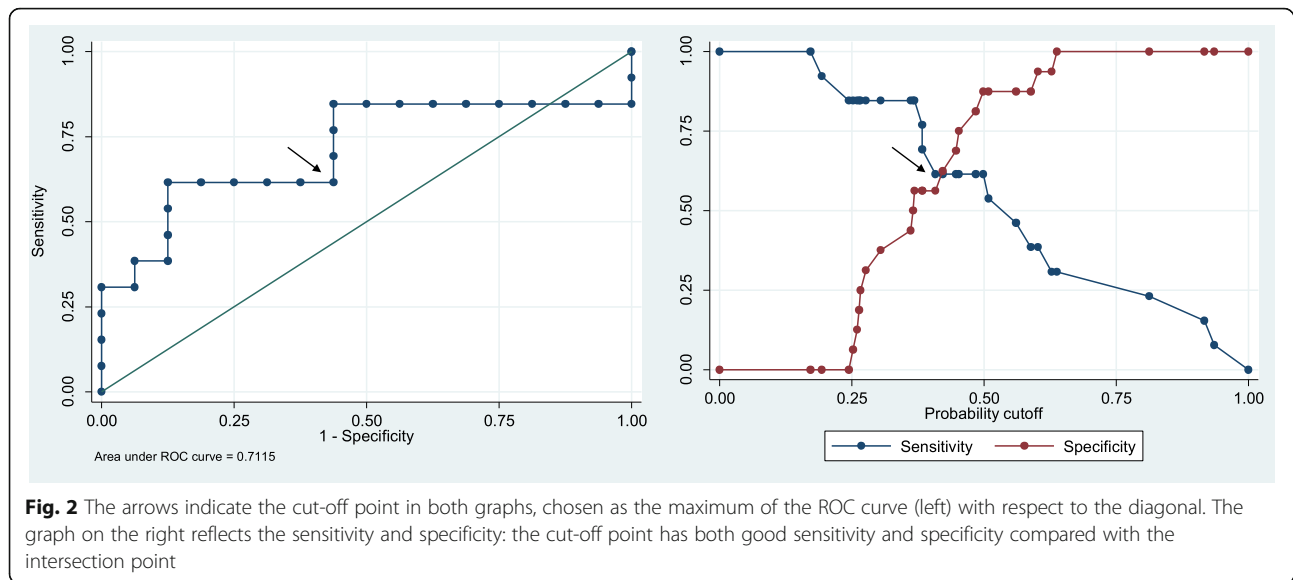
Discussion

In our cohort of patients, the circulating MMP-7 level, but not vascular ^{18}F -FDG-PET/CT focused on the carotid wall, proved to be capable of predicting cardiovascular events after a 1004-day follow-up, improving the performance of the traditional cardiovascular risk factors. This novel finding is consistent with data from a previous study [7] in which high plasma MMP-7 levels were independently associated with total mortality after a 3.5-year mean follow-up. In contrast with our study, the authors did not document an association with cardiovascular events [7]. In a previous study involving 106 endarterectomies, overexpression of the MMP-7 gene was shown to be 100-fold higher in plaques than in normal arteries [26].

The mechanisms underlying the association between MMP-7 and recurrent cardiovascular events have yet to be defined. One possible explanation is that MMP-7 may increase plaque vulnerability through inflammatory processes [8, 11], yet we did not find any correlation between circulating MMP-7 and in vivo carotid plaque inflammation assessed by ^{18}F -FDG-PET/CT. In low-inflammatory stages, ^{18}F -FDG PET/CT may be less sensitive than histology; however, we did not find a correlation between circulating and intraplaque MMPs. We could not measure intraplaque MMP-7 and thereby correlate this to its plasma level to show a direct relationship with carotid disease. The absence of a correlation between MMP levels and ^{18}F -FDG uptake in the carotid arteries, aortic arch or abdominal aorta suggests that high plasma level of MMP-7 may reflect the atherosclerosis burden but does not inform about the presence of plaque inflammation in a specific vascular territory. Additionally, arterial uptake of FDG is influenced by plaque composition. In line with our findings, the largest study to date evaluating the presence of vascular inflammation by ^{18}F -FDG-PET/MRI in individuals with atherosclerosis, showed that most FDG uptakes were detected in plaque-free arterial segments (suggesting an arterial inflammatory state at early stages of atherosclerosis) and in lipid-rich plaques [27]. Interestingly, previous studies performed in subjects with atherosclerotic disease did

Table 3 Correlation between plasma MMPs and histological findings

Histology	Plasma	Correlation	P-value
CD68	MMP-9	$r_s = 0.42$	0.009
	MMP-10	$r_s = 0.28$	0.09
	MMP-7	$r_s = 0.04$	0.83
	TIMP-1	$r_s = 0.38$	0.018
MMP-10	MMP-10	$r_s = 0.14$	0.45
MMP-9	MMP-9	$r_s = 0.37$	0.08
TIMP-1	TIMP-1	$r = 0.42$	0.02



not observe a relationship between markers of systemic inflammation and ^{18}F -FDG PET/CT in patients with carotid stenosis [11]. In the present study, all the samples corresponded to advanced calcified atherosclerotic plaques, in which an increase in calcium rarely overlaps with ^{18}F -FDG uptake [12]. A recent international multi-centre study showed that ^{18}F -FDG-PET was capable of predicting stroke recurrence although this study had a higher incidence of cerebrovascular events [28] compared to our study.

MMPs are not only produced in conjunction with inflammation. An *in vitro* study found that enriching smooth muscle cell cultures with collagen VIII stimulated MMP production, which induced cell migration [29]. Furthermore, the actions of MMPs are not only local. In fact, MMP-7 is also related to the development of hyperlipidaemia and atherosclerosis through its cleavage of lipid-free apolipoprotein A-IV, an event that plays an important role in lipid metabolism and oxidant activity [30]. All things considered, the presence of other inflammatory cell types cannot be excluded, as histological analysis detected CD68, which is expressed by phagocytes.

The presence of higher circulating levels of MMP-1 and MMP-10 in patients with carotid stenosis compared to healthy controls is in line with previous studies [31, 32], highlighting the relationship between atherosclerosis and MMPs. Surprisingly, circulating TIMP-1, believed to inhibit MMP action and contribute to plaque stability, had a weak to moderate correlation with ^{18}F -FDG-PET, probably due to the advanced stage of atherosclerosis.

The high rate of cardiovascular events we found can be explained by the extremely high prevalence of risk factors in our cohort and the systemic involvement of atherosclerosis. The presence of severe carotid stenosis has been

proposed as a risk factor for the development of stroke, white matter disease [33], coronary artery disease [34], peripheral artery disease and all-cause mortality [35]. Atherosclerosis is a systemic vascular disorder potentially involving multiple vascular territories. Our data suggest that MMP-7 may be an important mediator in the relationship between carotid stenosis and the development of recurrent cardiovascular events in different vascular territories. Nevertheless, the utility of MMP-7 as a potential biomarker may also be related to its role in other medical conditions, such as heart failure [36] as well as oncological diseases [37].

To date, several markers have been tested as potential tools to assess the risk of recurrent ischaemic stroke. The largest study tested 14 different biomarkers [38], including interleukin 6 and CRP [39], and limited utility in stroke prediction was found. Osteopontin, neopterin and myeloperoxidase [40], as well as lipoprotein-associated phospholipase A2 [41], free fatty acid levels [42] and circulating endothelial progenitor cells [43], have all been tested as recurrent stroke predictors with positive results, whereas the data regarding soluble CD40 ligand are inconsistent [40, 44]. Similar to MMP-7 in the present study, elevated lipoprotein (a) in patients with acute first ischaemic stroke [45] has been associated with a higher risk of combined recurrent cardiovascular events. However, none of these markers has been compared to MMP-7 in a similar setting, and therefore, larger studies including such patients should be performed.

The main strengths of this study are the detailed characterization of carotid plaque inflammation *in vivo* and the prolonged follow-up, with no loss. The main limitation of the study is the small number of patients, as larger studies may have identified differences in MMP-7 between symptomatic and asymptomatic patients or the utility of

the other potential biomarkers. Indeed, the consequent lack of statistical power may have hindered the discovery of the potential predictive value of ^{18}F -FDG-PET in cardiovascular events. In the histological study, the extensive decalcification required for the analysis of the endarterectomy samples may adversely affect tissue antigenicity, particularly that of extracellular proteins such as MMPs. Finally, peripheral artery disease is common in patients with carotid stenosis and we have not studied the presence of subclinical peripheral artery disease using ankle-brachial index, ultrasound, CT or MRI before endarterectomy [46]. Although our patients did not have symptoms of intermittent claudication, the presence of subclinical peripheral artery disease before endarterectomy cannot be completely excluded.

Conclusions

Our findings provide evidence that circulating (plasma) MMP-7 could represent a novel marker for recurrent cardiovascular events in patients with severe carotid stenosis. While additional studies will determine the value of MMP-7 in clinical practice, our results support the potential utility of circulating MMP-7 as a risk biomarker over and above that of conventional cardiovascular risk factors. To validate and confirm these results, larger studies with more statistical power should be carried out.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12872-020-01387-3>.

Additional file 1: Table IS. Semiquantitative visual grading scale to assess immunostaining (0–5 scale). **Table IIS.** Correlations between blood levels of MMPs and ^{18}F -FDG uptake.

Additional file 2: Figures 1S, 2S, 3S and 4S. Representative images corresponding to the semiquantitative visual grading scale used to assess the immunostaining for CD-68, TIMP-1, MMP-9 and MMP-10.

Abbreviations

^{18}F -FDG PET/CT: ^{18}F Fluorine-fluorodeoxyglucose-positron emission tomography combined with computed tomography; AHA: American Heart Association; ESR: Erythrocyte sedimentation rate; HDL: High-density lipoprotein; hs-CRP: High-sensitivity C-reactive protein; LDL: Low-density lipoprotein cholesterol; MMPs: Metalloproteinases; NASCET: North American Symptomatic Carotid Endarterectomy Trial; RICORNA: Risk score for coronary events in the population of Navarra; ROC: Receiver operating characteristic; SUVmax: Standardized uptake value; TBR: Target-to-background ratio; TIA: Transient ischaemic attack; TIMP-1: Tissue inhibitor of matrix metalloproteinase 1

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Authors' contributions

Conception and design: DMA, PI, EMV, JGC. Analysis and interpretation: DMA, PI, JAR, JLF, EMV. Data collection: DMA, PI, JAR. Writing the article: DMA, PI, JAR, MJGV, JLF, PD. Critical revision of the article: PI, JAR, MJGV, JLF, LFA, LG, RM, PD, JGC, EMV. Final approval of the article: DMA, PI, JAR, MJGV, JLF, LFA, LG, RM, PD, JGC, EMV. Statistical analysis: DMA, JLF. Obtained funding: PI,

EMV, Overall responsibility: PI, EMV. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

All protocols were approved by the Committee for Medical and Research Ethics, *Comité de ética de la Investigación* of the University of Navarra. Project: 058/2012. Participants gave written informed consent. Samples from patients included in the study were provided by the Biobank of the University of Navarra and were processed following standard operating procedures approved by the Research Ethics and Scientific Committees.

Consent for publication

Written informed consent for publication was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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References

1. Ay H, Furie KL, Singhal A, Smith WS, Sorensen AG, Koroshetz WJ. An evidence-based causative classification system for acute ischemic stroke. *Ann Neurol*. 2005;58(5):688–97.
2. Vilarino-Rico J, Pita-Fernandez S, Segura-Iglesias RJ. Clinical predictors of major adverse cardiovascular events during long-term follow-up after carotid endarterectomy. *Ann Vasc Surg*. 2015;29(3):419–25.
3. Avgerinos ED, Go C, Ling J, Makaroun MS, Chaer RA. Survival and long-term cardiovascular outcomes after carotid endarterectomy in patients with chronic renal insufficiency. *Ann Vasc Surg*. 2015;29(1):15–21.
4. Nam HJ, Heo SH, Kim BJ, San Lee J, Youn HC, Lee JS, et al. Long-term outcome after Carotid Endarterectomy in patients with ischemic heart disease. *World Neurosurg*. 2018;110:e806–e14.
5. Castillo J, Alvarez-Sabin J, Martinez-Vila E, Montaner J, Sobrino T, Vivancos J, et al. Inflammation markers and prediction of post-stroke vascular disease recurrence: the MITICO study. *J Neurol*. 2009;256(2):217–24.
6. Rodriguez JA, Orbe J, Paramo JA. Metalloproteinases, vascular remodeling and atherothrombotic syndromes. *Rev Esp Cardiol*. 2007;60(9):959–67.
7. Abbas A, Aukrust P, Russell D, Krohg-Sorensen K, Almas T, Bundgaard D, et al. Matrix metalloproteinase 7 is associated with symptomatic lesions and adverse events in patients with carotid atherosclerosis. *PLoS One*. 2014;9(11):e84935.
8. Chistiakov DA, Sobenin IA, Orekhov AN. Vascular extracellular matrix in atherosclerosis. *Cardiol Rev*. 2013;21(6):270–88.

9. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res*. 2003; 92(8):827–39.
10. Hansson J, Vasan RS, Arnlov J, Ingelsson E, Lind L, Larsson A, et al. Biomarkers of extracellular matrix metabolism (MMP-9 and TIMP-1) and risk of stroke, myocardial infarction, and cause-specific mortality: cohort study. *PLoS One*. 2011;6(11):e16185.
11. Duivenvoorden R, Mani V, Woodward M, Kallend D, Suchankova G, Fuster V, et al. Relationship of serum inflammatory biomarkers with plaque inflammation assessed by FDG PET/CT: the dal-PLAQUE study. *JACC Cardiovasc Imaging*. 2013;6(10):1087–94.
12. Rudd JH, Myers KS, Bansilal S, Machac J, Woodward M, Fuster V, et al. Relationships among regional arterial inflammation, calcification, risk factors, and biomarkers: a prospective fluorodeoxyglucose positron-emission tomography/computed tomography imaging study. *Circ Cardiovasc Imaging*. 2009;2(2):107–15.
13. Mahdessian H, Perisic Matic L, Lengquist M, Gertow K, Sennblad B, Baldassarre D, et al. Integrative studies implicate matrix metalloproteinase-12 as a culprit gene for large-artery atherosclerotic stroke. *J Intern Med*. 2017;282(5):429–44.
14. Wu YW, Kao HL, Huang CL, Chen MF, Lin LY, Wang YC, et al. The effects of 3-month atorvastatin therapy on arterial inflammation, calcification, abdominal adipose tissue and circulating biomarkers. *Eur J Nucl Med Mol Imaging*. 2012;39(3):399–407.
15. von Reutern GM, Goertler MW, Bornstein NM, Del Sette M, Evans DH, Hetzel A, et al. Grading carotid stenosis using ultrasonic methods. *Stroke*. 2012; 43(3):916–21.
16. North American Symptomatic Carotid Endarterectomy Trial C, Barnett HJM, Taylor DW, Haynes RB, Sackett DL, Peerless SJ, et al. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. *N Engl J Med*. 1991;325(7):445–53.
17. Kratz A, Plebani M, Peng M, Lee YK, McCafferty R, Machin SJ, et al. ICSH recommendations for modified and alternate methods measuring the erythrocyte sedimentation rate. *Int J Lab Hematol*. 2017;39(5):448–57.
18. Orbe J, Montero I, Rodriguez JA, Beloqui O, Roncal C, Paramo JA. Independent association of matrix metalloproteinase-10, cardiovascular risk factors and subclinical atherosclerosis. *J Thromb Haemost*. 2007;5(1):91–7.
19. Diener HC, Bogousslavsky J, Brass LM, Cimminiello C, Csiba L, Kaste M, et al. Aspirin and clopidogrel compared with clopidogrel alone after recent ischaemic stroke or transient ischaemic attack in high-risk patients (MATCH): randomised, double-blind, placebo-controlled trial. *Lancet*. 2004;364(9431):331–7.
20. Layden J, Michaels J, Birmingham S, Higgins B, Guideline DG. Diagnosis and management of lower limb peripheral arterial disease: summary of NICE guidance. *BMJ*. 2012;345:e4947.
21. Aboyans V, Ricco JB, MEL B, Bjorck M, Brodmann M, Cohnert T, et al. 2017 ESC Guidelines on the Diagnosis and Treatment of Peripheral Arterial Diseases, in collaboration with the European Society for Vascular Surgery (ESVS): Document covering atherosclerotic disease of extracranial carotid and vertebral, mesenteric, renal, upper and lower extremity arteries Endorsed by: the European Stroke Organization (ESO) The Task Force for the Diagnosis and Treatment of Peripheral Arterial Diseases of the European Society of Cardiology (ESC) and of the European Society for Vascular Surgery (ESVS). *Eur Heart J*. 2018;39(9):763–816.
22. Patel MR, Bailey SR, Bonow RO, Chambers CE, Chan PS, Dehmer GJ, et al. ACCF/SCAI/AATS/AHA/ASE/ASNC/HFSA/HRS/SCCM/SCCT/SCMR/STS 2012 appropriate use criteria for diagnostic catheterization: American College of Cardiology Foundation Appropriate Use Criteria Task Force Society for Cardiovascular Angiography and Interventions American Association for Thoracic Surgery American Heart Association, American Society of Echocardiography American Society of Nuclear Cardiology Heart Failure Society of America Heart Rhythm Society, Society of Critical Care Medicine Society of Cardiovascular Computed Tomography Society for Cardiovascular Magnetic Resonance Society of Thoracic Surgeons. *Catheter Cardiovasc Interv*. 2012;80(3):E50–81.
23. Figueroa AL, Abdelbaky A, Truong QA, Corsini E, MacNabb MH, Lavender ZR, et al. Measurement of arterial activity on routine FDG PET/CT images improves prediction of risk of future CV events. *JACC Cardiovasc Imaging*. 2013;6(12):1250–9.
24. Gonzalez-Diego P, Moreno-Iribas C, Guembe MJ, Vines JJ, Vila J. Adaptation of the Framingham-Wilson coronary risk equation for the population of Navarra (RICORNA). *Rev Esp Cardiol*. 2009;62(8):875–85.
25. Stary HC. Natural history and histological classification of atherosclerotic lesions: an update. *Arterioscler Thromb Vasc Biol*. 2000;20(5):1177–8.
26. Razuvaev A, Ekstrand J, Folkersen L, Agardh H, Markus D, Swedenborg J, et al. Correlations between clinical variables and gene-expression profiles in carotid plaque instability. *Eur J Vasc Endovasc Surg*. 2011;42(6):722–30.
27. Fernandez-Friera L, Fuster V, Lopez-Melgar B, Oliva B, Sanchez-Gonzalez J, Macias A, et al. Vascular inflammation in subclinical atherosclerosis detected by hybrid PET/MRI. *J Am Coll Cardiol*. 2019;73(12):1371–82.
28. Kelly PJ, Camps-Renom P, Giannotti N, Marti-Fabregas J, Murphy S, McNulty J, et al. Carotid plaque inflammation imaged by (18)F-Fluorodeoxyglucose positron emission tomography and risk of early recurrent stroke. *Stroke*. 2019;50(7):1766–73.
29. Hou G, Mulholland D, Gronska MA, Bendeck MP. Type VIII collagen stimulates smooth muscle cell migration and matrix metalloproteinase synthesis after arterial injury. *Am J Pathol*. 2000;156(2):467–76.
30. Park JY, Park JH, Jang W, Hwang IK, Kim UJ, Kim HJ, et al. Apolipoprotein A-IV is a novel substrate for matrix metalloproteinases. *J Biochem*. 2012;151(3):291–8.
31. Alvarez B, Ruiz C, Chacon P, Alvarez-Sabin J, Matas M. Serum values of metalloproteinase-2 and metalloproteinase-9 as related to unstable plaque and inflammatory cells in patients with greater than 70% carotid artery stenosis. *J Vasc Surg*. 2004;40(3):469–75.
32. Martinez-Aguilar E, Gomez-Rodriguez V, Orbe J, Rodriguez JA, Fernandez-Alonso L, Roncal C, et al. Matrix metalloproteinase 10 is associated with disease severity and mortality in patients with peripheral arterial disease. *J Vasc Surg*. 2015;61(2):428–35.
33. Kandiah N, Goh O, Mak E, Marmin M, Ng A. Carotid stenosis: a risk factor for cerebral white-matter disease. *J Stroke Cerebrovasc Dis*. 2014;23(1):136–9.
34. Hamada S, Kashiwazaki D, Yamamoto S, Akioka N, Kuwayama N, Kuroda S. Impact of plaque composition on risk of coronary artery diseases in patients with Carotid artery stenosis. *J Stroke Cerebrovasc Dis*. 2018;27(12):3599–604.
35. Zhang Y, Fang X, Hua Y, Tang Z, Guan S, Wu X, et al. Carotid artery plaques, Carotid intima-media thickness, and risk of cardiovascular events and all-cause death in older adults: a 5-year prospective, Community-Based Study. *Angiology*. 2018;69(2):120–9.
36. Cuvelliez M, Vandewalle V, Brunin M, Beseme O, Hulot A, de Groote P, et al. Circulating proteomic signature of early death in heart failure patients with reduced ejection fraction. *Sci Rep*. 2019;9(1):19202.
37. Klupp F, Neumann L, Kahlert C, Diers J, Halama N, Franz C, et al. Serum MMP7, MMP10 and MMP12 level as negative prognostic markers in colon cancer patients. *BMC Cancer*. 2016;16:494.
38. Segal HC, Burgess AI, Poole DL, Mehta Z, Silver LE, Rothwell PM. Population-based study of blood biomarkers in prediction of subacute recurrent stroke. *Stroke*. 2014;45(10):2912–7.
39. Singh TP, Morris DR, Smith S, Moxon JV, Golledge J. Systematic review and meta-analysis of the association between C-reactive protein and major cardiovascular events in patients with peripheral artery disease. *Eur J Vasc Endovasc Surg*. 2017;54(2):220–33.
40. Ganz P, Amarenco P, Goldstein LB, Silleisen H, Bao W, Preston GM, et al. Association of Osteopontin, Neopterin, and myeloperoxidase with stroke risk in patients with prior stroke or transient ischemic attacks: results of an analysis of 13 biomarkers from the stroke prevention by aggressive reduction in cholesterol levels Trial. *Stroke*. 2017;48(12):3223–31.
41. Wei L, Ke Z, Zhao Y, Cai Z. The elevated lipoprotein-associated phospholipase A2 activity is associated with the occurrence and recurrence of acute cerebral infarction. *Neuroreport*. 2017;28(6):325–30.
42. Niu Z, Hu H, Tang F. High free fatty acid levels are associated with stroke recurrence and poor functional outcome in Chinese patients with ischemic stroke. *J Nutr Health Aging*. 2017;21(10):1102–6.
43. Marti-Fabregas J, Delgado-Mederos R, Crespo J, Pena E, Marin R, Jimenez-Xarrie E, et al. Circulating endothelial progenitor cells and the risk of vascular events after ischemic stroke. *PLoS One*. 2015;10(4):e0124895.
44. Li J, Wang Y, Lin J, Wang D, Wang A, Zhao X, et al. Soluble CD40L is a useful marker to predict future strokes in patients with minor stroke and transient ischemic attack. *Stroke*. 2015;46(7):1990–2.
45. Lange KS, Nave AH, Liman TG, Grittner U, Endres M, Ebinger M. Lipoprotein(a) levels and recurrent vascular events after first ischemic stroke. *Stroke*. 2017; 48(1):36–42.

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