Calprotectin predicts mortality after ischemic stroke and is present in the thrombus

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Research

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Abstract

**Background**- Inflammatory response plays an important role in many processes related to acute ischemic stroke (AIS). Calprotectin (S100A8/S100A9), released by monocytes and neutrophils, is a key protein in the regulation of inflammation and thrombosis. The purpose of this study is to evaluate the association of circulating calprotectin with other inflammatory biomarkers and AIS prognosis, as well as the calprotectin content in stroke thrombi.

**Methods**- Among the 748 patients treated at a comprehensive stroke centre between 2015-2017, 413 patients with confirmed acute ischemic injury were evaluated. Patients with systemic inflammation or infection at onset were excluded. Plasma calprotectin was measured by ELISA in blood samples of AIS patients within the first 24h. Univariate and multivariate logistic regression models were performed to evaluate its association with mortality and functional independence (FI) at 3-months (defined as modified Rankin Scale<2), and intracranial haemorrhage (ICH) after stroke. Further, S100A9 was localized by immunostaining in stroke thrombi (n=44).

**Results**- Higher calprotectin levels were associated with 3-month mortality and ICH, while lower calprotectin levels were documented in patients with 3-month FI. After adjusting for potential confounders, plasma calprotectin levels remained associated with 3-month mortality [OR (95%CI); 3.06, (1.67-5.61)]. Patients with calprotectin ≥ 2.26 µg/mL were 4-times more likely to die [OR 3.98, (1.88-8.41)]. Likewise, Neutrophil-Lymphocyte Ratio (NLR) and C-Reactive Protein (CRP) were also associated with 3-month mortality [OR 1.98, (1.17-3.35); and 1.39, (1.02-1.89) respectively]. A multimarker approach demonstrated that patients with increased calprotectin, CRP and NLR had the poorest outcome with a mortality rate of 42.3% during follow-up. S100A9 protein, as part of the heterodimer calprotectin, was present in all thrombi retrieved from AIS patients. Mean S100A9 content was 3.5% and tended to be higher in patients who died (p=0.09). Moreover, it positively correlated with platelets (Pearson r 0.46, p<0.002); leukocytes (0.45, p<0.01) and neutrophil elastase (0.70, p<0.001) thrombi content.

**Conclusions**- Plasma calprotectin is an independent predictor of 3-month mortality and provides complementary prognostic information to identify patients with poor outcome after AIS. Presence of S100A9 in stroke thrombi suggests a possible inflammatory mechanism in clot formation and further studies are needed to determine its influence in resistance to reperfusion.

Introduction

Stroke is the most frequent cause of permanent disability in adults and one of the most important causes of death [1]. Over the next years, global stroke burden is expected to increase steadily, mainly because of population aging [2]. Ischemic stroke remains to represent more than 80% of all strokes. Even after years of research, pathophysiology of brain ischemia and post-ischemic changes in the brain are not yet fully understood. Inflammation is increasingly recognized as a key element in pathological progression of ischemic stroke. Likely, multiple inflammatory molecules have been reported as predictive markers of
stroke severity and outcome, and proposed as potential therapeutic targets to be modulated as a neuroprotective strategy in acute ischemic stroke (AIS) [3].

Neutrophil-to-lymphocyte ratio (NLR), easily calculated by white blood cell count on admission, has been reported as a prognostic biomarker in stroke. High admission NLR values predict 3-month clinical outcome in thrombectomised patients [4] and are associated with symptomatic intracranial hemorrhage (ICH) [5]. C-Reactive Protein (CRP), a sensitive indicator of inflammation rapidly produced by the liver after tissue injury or infection [6], is one of the most widely used marker in clinical practice. In AIS patients, CRP levels have been reported to be significantly higher than controls in all ischaemic stroke subtypes [7].

Calprotectin is a heterodimer formed by two cytosolic proteins, S100A8 and S100A9, expressed by white blood cells, especially monocytes and neutrophils. Also known as myeloid-related protein-8 and -14 (MRP8/14), calprotectin plays an important role in promoting inflammation and is a validated marker of disease activity in inflammatory bowel disease and rheumatoid arthritis [8]. Studies focusing on coronary events have shown that plasma calprotectin was related to first and future coronary events, independently of traditional cardiovascular risk factors and C-reactive protein [9]. Recently, circulating calprotectin was associated with increased peripheral artery disease risk prediction, further improved when combined with high sensitivity CRP [10]. Moreover, plasma calprotectin has been proposed as a diagnostic marker of AIS [11] and, inhibition of S100A9 has been reported to suppress thrombus formation in experimental models of stroke [12,13].

Nevertheless, calprotectin prognostic role and its association with other inflammatory biomarkers, such as CRP and NLR, have not yet been studied in stroke patients. The purpose of this study is to evaluate circulating calprotectin levels as a prognosis biomarker in AIS and to analyse how it relates to other well-known inflammatory biomarkers. Furthermore, we have studied the composition of ischemic stroke thrombi and characterised S100A9 content, to assess its association with thrombi components and clinical parameters.

**Methods**

**Study population**

This is a single-centre retrospective analysis of consecutive, prospectively collected, AIS patients admitted to our Stroke Unit of the Complejo Hospitalario de Navarra between November 2015 and November 2017. All patients with neurologic deficits were included when AIS was suspected and written informed consent was obtained (n=748). Patients with transient ischemic attack and stroke mimics were excluded and only those with established acute ischemic injury confirmed by neuroimaging (mainly Diffusion Weighted-MRI and some of them delayed cranial CT) were included in this study. Patients with systemic inflammation or infection at onset (including active cancer, post-operative patients and active systemic infections) were excluded to avoid confusion with previous inflammatory condition (Figure 1).
Clinical information

Baseline characteristics of the study patients, including demographics (age, sex), previous cardiovascular disease, vascular risk factors, serum glucose, systolic and diastolic blood pressure (SBP and DBP respectively) at admission, previous use of antithrombotic agents (antiplatelet agent and anticoagulants), stroke severity assessed by National Institutes of Health Stroke Scale (NIHSS) and treatment with tissue-type plasminogen activator (tPA) and/or mechanical thrombectomy, were recorded. The vascular risk factors included were: hypertension (patients taking antihypertensive drugs or with blood pressure >140/90 mmHg on repeated measurements), type-2 diabetes (patients taking antidiabetic drugs, fasting blood sugar ≥ 126 mg/dL or HbA1c ≥ 6.5%, or a casual plasma glucose >200 mg/dL), hypercholesterolemia [patients receiving lipid-lowering agents or with an overnight fasting cholesterol level ≥ 240 mg/dL, triglycerides ≥ 200 mg/dL, or low-density lipoprotein (LDL) cholesterol ≥ 160mg/dL], and current cigarette smoking. Etiologic subtypes of ischemic stroke were classified based on the Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification [14].

A CT scan was obtained at admission for all patients and the Alberta Stroke Program Early CT Score (ASPECTS) was collected by two independent radiologists. CT scan was repeated at 24-48 hours in those patients who underwent mechanical thrombectomy and/or received tPA in order to evaluate infarct area and identify ICH. MRI scans were obtained within one week of stroke onset using a 1.5 T MR imaging unit if not contraindicated (delayed CT scan was elective when MRI was contraindicated). ICH after AIS was identified on this follow-up neuroimaging and characterized by a stroke specialized neurologist.

Samples processing and plasma calprotectin measurement

Venous blood samples were drawn from all patients in the emergency department before the initiation of any treatment as standard-of-care and neutrophils, lymphocytes (Alinity hq, Abbott, USA), and serum glucose (Architect i2000SR, Abbott, USA) were measured by standard laboratory techniques. After receiving or not treatment, new blood samples were taken within the following 24h and C-reactive protein (CRP) was measured with an autoanalyzer (Architect i2000SR, Abbott, USA). These second samples were centrifuged at 1200 xg for 15 minutes, 4 °C within 2 hours of collection and stored at -80°C for further analysis. Citrated plasma samples were thawed on ice and thoroughly vortexed before measuring calprotectin levels (LEGEND MAX Human MRP8/14 ELISA Kit, BioLegend, USA) with an automated ELISA analyzer TRITURUS (Grifols, Spain). The inter- and intra-assay variability averages were 7.4% and 2.8% respectively. All experiments were performed in a blinded manner following manufacturer’s instructions.

Histological analysis

Retrieved thrombi from thrombectomised IS patients (n=44) were transferred to saline until fixed in formalin (PanReac AppliChem, Spain) during 24h. Then, samples were embedded in paraffin by a tissue automatic processor (Tissue-Tek VIP, Sakura, Japan) and paraffin-embedded clot material was cut into 3
µm sections with a rotatory microtome (HM-340E, Microm, Germany). Serial slides from each thrombus were stained with hematoxylin and eosin (PanReac AppliChem, Spain), with Martius Scarlet Blue staining (Atom, UK), and with specific antibodies against platelets (CD42b, Invitrogen, USA), neutrophil elastase (NE, Sigma-Aldrich, USA) and the calcium-binding protein S100A9 (PA5-82145, Invitrogen, USA) as part of the heterodimer calprotectin. Immunostained slides were scanned (Aperio ImageScope, Leica ByoSistems, Germany) and quantified with ImageJ software [15]. Data are presented as the percentage of positive stained area in total tissue area.

**Outcome measures**

Main clinical outcomes were defined by modified Rankin Scale (mRS) score at 90 days, established by face-to-face interview with a stroke specialized neurologist. Primary clinical outcome was 3-month all-cause mortality. Secondary outcomes included 3-month-functional independence, defined as 90-day mRS <3, and ICH after ischemic stroke including hemorrhagic infarcts (HI type 1 or 2), parenchymal hematomas (PH type 1 or 2) and remote hematomas or subarachnoid hemorrhages, according to the European Cooperative Acute Stroke Study III (ECASS III) classification [16]. Patients without follow-up regarding stroke related functional outcome after 90 days were excluded from the analysis for FI.

**Statistical analysis**

Continuous variables with normal distributions were presented as mean with standard deviation (SD), while non-normally distributed variables were presented as median with interquartile range (IQR). Normality of distributions was assessed graphically and with the Shapiro-Wilk test. Logarithmic transformation was applied for continuous variables with skewed distributions. Continuous variables were compared between groups using the unpaired t-test or the Wilcoxon rank-sum test depending on their distribution. Comparisons between binary categorical variables were performed using the Chi-square test or, in the case of small-expected frequencies, Fisher’s exact test. Pairwise Spearman correlations were performed between continuous variables to evaluate correlation. Stroke subtype classification was assessed by TOAST criteria and dichotomized etiological groups were created. NIHSS score was categorized ([0-7], [7-14] and [>14]) and analysis of variance and trend analysis were performed.

Association of baseline characteristics with outcomes was performed first with univariate logistic regression models. To evaluate 3-month functional independence (mRS <3), patients with an initial qualifying status (mRS score >2) were excluded (n=50). To select independent predictors to include in each model, baseline characteristics associated with a value of p<0.20 in univariate analyses were implemented in a forward-stepwise multivariate logistic regression model with a significance level for addition to the model of p<0.05. Results were expressed as odds ratios (ORs) with 95% confidence intervals (95% CI). The Homer-Lemeshow test was used to assess calibration of the models. K-fold cross-validation was performed to test the model's ability to predict new data that was not used in estimating it [17].
In addition, potential confounders were included if their inclusion led to clinically relevant changes (OR variations >±10%). Interactions were evaluated with the likelihood-ratio test and stratified models were generated if the rendered p values were below 0.05. Selected multivariate binary logistic regression models were performed to evaluate associations between calprotectin with clinical outcomes after adjustment for potential confounders.

Receiver operating characteristic (ROC) curve analysis, with binary logistic regression, was used to determine the discrimination capacity of calprotectin, NIHSS, CRP, and NLR as outcome predictors. ROC analysis was used to compare the complete model for each outcome selected by forward stepwise with and without calprotectin. Besides, a cut-off point for each of the inflammatory markers tested (NLR, CRP and calprotectin) was rendered by ROC analysis and mortality predictions were performed according to the presence of none, 1, 2 or 3 increased markers as defined by these cut-offs.

For all analyses p<0.05 was considered statistically significant. All analyses were performed with the STATA software (version 14.2, StataCorp LLC, Texas, USA).

Results

A total of 413 patients with AIS confirmed by neuroimaging were finally included for the analysis. The mean age of our population was 75.6 years (SD: 0.6 years), 71.4% were hypertensive and 27.1% diabetic. Among this elderly population, around 11.9% of patients had baseline mRS score ≥3. Stroke severity was mild with median NIHSS score of 6 points (IQR: 3-15). ICH incidence was 12.4% and mortality rate was 17.1%. Baseline characteristics of the study patients are presented in Table 1.

Table 1. Baseline characteristics of the study population
| Variable                                      | n=413 |
|----------------------------------------------|-------|
| Age; years *                                  | 75.6 (0.6) |
| Female ‡                                      | 176 (42.6) |
| Hypertension ‡                                | 295 (71.4) |
| Type-2 Diabetes ‡                            | 112 (27.1) |
| Dyslipidemia ‡                                | 201 (48.7) |
| Antiplatelet therapy ‡                       | 129 (31.2) |
| Anticoagulant therapy ‡                      | 86 (20.8) |
| SBP at admission, mmHg ‡                     | 152 (135-171) |
| DBP at admission, mmHg ‡                     | 82 (73-93) |
| Serum glucose at admission, mg/dl ‡          | 113 (99.5-142) |
| Neutrophil count at admission, x10⁹/L ‡      | 5.2 (4.1-6.7) |
| Lymphocyte count at admission, x10⁹/L ‡      | 1.8 (1.3-2.4) |
| Baseline NIHSS score ‡                       | 6 (3-15) |
| ASPECTS <7 ‡                                  | 16 (4.01) |
| ASPECTS, points *                             | 9.45 (1.24) |
| Baseline mRS score ‡                          |       |
| mRS 0                                        | 164 (39.8) |
| mRS 1                                        | 125 (30.3) |
| mRS 2                                        | 74 (18.0) |
| mRS 3                                        | 32 (7.8) |
| mRS 4                                        | 17 (4.1) |
| Intravenous thrombolysis ‡                   | 151 (36.6) |
| Endovascular treatment ‡                     | 70 (17.0) |
| Intracranial hemorrhage ‡                    | 51 (12.4) |
| 3-month mortality ‡                          | 67 (17.1) |
| 3-month functional independence ‡            | 227 (57.9) |

SD: standard deviation; IQR: interquartile range (denoted by 25th-75th percentile); SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; NIHSS: National Institute of Health Stroke Scale; ASPECTS:
Alberta Stroke Program Early CT Score; mRS: modified Rankin Scale.

* Continuous variables with normal distributions are presented as means (SD).

† Continuous non-normally distributed variables are presented as medians (IQR).

‡ Categorical variables are presented as n (%).

The most common etiology by TOAST was cardioembolic (n=178, 43%), followed by small-vessel occlusion (n=62, 15%), large artery atherosclerosis (n=43, 10.4%) and others causes (n=7, 1.7%) with 30% of strokes with undetermined etiology (n=123).

**Plasma calprotectin levels by outcomes**

Median plasma calprotectin level was 1.76 µg/mL (IQR, 1.14-2.66). As shown in Figure 2A, patients who died within 3 months showed higher calprotectin levels [median, (IQR) µg/mL; 2.81, (2.06-4.26) vs 1.58, (1.00-2.29), p<0.001]; as did patients who developed ICH after AIS [1.99, (1.36-3.32) vs 1.72, (1.10-2.56), p=0.030, Figure 2B]. Furthermore, lower plasma calprotectin levels after AIS were observed in patients with 3-month FI [1.49, (0.97-2.23) vs 2.2, (1.41-3.38), p<0.001, Figure 2C].

[Insert Figure 2]

**Relationship between calprotectin and clinical and analytical variables**

Plasma calprotectin levels correlated with neutrophil (Pearson r, 0.28, p<0.001) and lymphocyte count (-0.17, p=0.007); NLR (0.29, p<0.001); CRP (0.50, p<0.001) and fibrinogen (0.27, p=0.001).

Calprotectin levels were significantly associated with clinical stroke severity by NIHSS score in the ANOVA test (p <0.001) and a significant linear trend was observed (p <0.001) (Additional file 1: Supplementary Figure 1A).

When analysing clinical factors, a positive correlation with age was registered (Pearson r, 0.18, p<0.001) with a significant linear trend (p=0.012) in the ANOVA test. In addition, mRS score at baseline was also associated with plasma calprotectin levels (p<0.02,) with a significant positive linear trend (p<0.001).

Patients with confirmed cardioembolic stroke -according to TOAST criteria- showed higher calprotectin levels than patients with non-cardioembolic stroke [1.97, (1.23-2.97) vs 1.66, (0.99-2.39); p<0.004] (Additional file 1: Supplementary Figure 1B).

**Predictors of mortality at 90 days**

Patients who died within 90 days were older (median age, 86.6 vs 75.7 years ; p<0.001), more frequently females (58.2 vs 41.8% ; p=0.05), had higher DBP (median DBP, 87.5 vs 82.9 mmHg; p=0.04), had worse
NIHSS score (median NIHSS score, 17 vs 4; p<0.001), mRS at admission (median mRS, 1.94 vs 0.91; p<0.001), and a lower ASPECTS (median ASPECT score, 8.5 vs 9.6; p<0.001) than patients who survived at 90 days.

Univariate analysis confirms sex, age, DBP, NIHSS score, baseline mRS and ASPECT score association with mortality (Table 2). In addition, intravenous treatment with tPA was associated with a higher 3-month mortality and a higher incidence of ICH after stroke. Elevated inflammatory markers (neutrophil count, NLR, CRP and calprotectin) were associated with a higher risk of mortality. After adjusting for potential confounders, plasma calprotectin levels remained associated with 3-month mortality [per a log+1 increase OR, (95% CI); 3.06, (1.67-5.61)], in a model adjusted by age, glucose at admission, stroke severity by NIHSS score, ASPECTS, baseline mRS score, and intravenous thrombolysis. Similarly, NLR and CRP also remained associated with 3-month mortality with the same adjustment [per a log+1 increase: OR 1.98, (1.17-3.35); and 1.39, (1.02-1.89) respectively].

As shown in Table 2, after forward stepwise selection of the strongest predictors, only age [per 10-year increase: OR, (95% CI); 1.73, (1.18-2.53)], calprotectin [per a log+1 increase: OR 3.00 (1.71-5.26)], NIHSS score >14 [OR 7.04, (3.08-16.10)], ASPECTS >7 [OR 0.19 (0.05-0.77)] and baseline mRS score [OR 1.57 (1.14-2.15)] were independent predictors of 3-month mortality, whereas NLR and CRP were excluded from the analysis (p>0.05). This predictive model had a good calibration as indicated by the Hosmer-Lemeshow goodness-of-fit test (p value= 0.51). The averaged measure of fitness in k-fold cross-validation with k=5 was 0.30 for this model (range 0.23-0.37).

**Table 2.** Univariate and multivariate logistic regression models of 3-month mortality with calprotectin and other baseline characteristics.
| Variable | Univariate Logistic Regression | Multivariate Logistic Regression (n=393) |
|----------|-------------------------------|----------------------------------------|
|          | OR (95%CI)                    | p value | OR (95%CI) | p value |
| Age (per 10-year increase) | 2.21 (1.65-2.97) | <0.001 | 1.73 (1.18-2.53) | 0.005 |
| Sex (female) | 2.09 (1.23-3.56) | 0.007 | | |
| Hypertension | 2.95 (1.41-6.18) | 0.004 | | |
| Type-2 Diabetes | 0.64 (0.34-1.21) | 0.168 | | |
| Dyslipidaemia | 0.66 (0.39-1.13) | 0.131 | | |
| SBP at admission * (per 10mmHg increase) | 2.35 (0.48-11.48) | 0.290 | | |
| DBP at admission (per 10mmHg increase) | 1.17 (1.00-1.36) | 0.048 | | |
| Serum Glucose (mg/dL) * | 1.97 (0.88-4.44) | 0.101 | | |
| Neutrophil count (x mm3) * | 3.64 (1.83-7.24) | <0.001 | | |
| Lymphocyte count (x mm3) * | 0.42 (0.24-0.72) | 0.002 | | |
| NLR * | 2.41 (1.63-3.58) | <0.001 | | |
| C-Reactive Protein * (mg/L) | 1.02 (1.01-1.03) | <0.001 | | |
| Calprotectin µg/mL * | 4.60 (2.86-7.40) | <0.001 | 3.00 (1.71-5.26) | <0.001 |
| NIHSS score | | | | |
| 7-14 (vs 0-7) | 3.70 (1.55-8.86) | 0.003 | 2.23 (0.83-6.02) | 0.112 |
| >14 (vs 0-7) | 11.86 (5.85-24.05) | <0.001 | 7.04 (3.08-16.10) | <0.001 |
| ASPECTS ≥7 | 0.08 (0.03-0.24) | <0.001 | 0.19 (0.05-0.77) | 0.020 |
| Intravenous thrombolysis | 2.33 (1.37-3.98) | 0.002 | | |
|                        | Value         | p-value |
|------------------------|---------------|---------|
| Endovascular treatment | 1.49 (0.77-2.89) | 0.240   |
| Intracranial Hemorrhage| 4.04 (2.10-7.77) | <0.001  |
| baseline mRS score     | 2.10 (1.66-2.65) | <0.001  | 1.57 (1.14-2.15) | 0.005 |

SBP: Systolic Blood Pressure; DBP: Diastolic Blood Pressure; NLR: Neutrophil-to-lymphocyte Ratio; NIHSS: National Institute of Health Stroke Scale; ASPECTS: Alberta Stroke Program Early CT Score; mRS: modified Rankin Scale.

* log transformed.

† Excluded by forward stepwise selection (inclusion p<0.05 and exclusion p>0.10)
‡ Excluded to avoid collinearity between Neutrophils, Lymphocytes and NLR.

ROC curves analysis for significant univariate models showed that the highest AUC was for NIHSS score [area under the curve (AUC), (95%CI): 0.83 (0.77-0.88), Figure 3A] followed by calprotectin as the strongest predictor of mortality across the inflammatory markers tested [AUC (95%CI): calprotectin 0.77 (0.71-0.83)]. The inclusion in the ROC analysis of age, serum glucose, NIHSS score, baseline mRS, intravenous thrombolysis and ASPECTS as covariates, rendered an AUC for 3-month mortality of 0.88 (95%CI: 0.84-0.93; Figure 3B), that was significantly improved when calprotectin was included in the model [AUC (95%CI): 0.90 (0.86-0.94); p=0.04].

ROC analysis for calprotectin rendered a cut-off value of 2.26µg/mL (sensitivity: 72.7%; specificity: 74.3%), which showed a negative predictive value of 92.9% in our population. When stratifying by this optimal cut-off and controlling for age, serum glucose, NIHSS score, baseline mRS, intravenous tPA administration and ASPECTS; patients with calprotectin ≥ 2.26µg/mL were 4 times more likely to die within 90 days than AIS patients with a calprotectin < 2.26µg/mL [OR, (95%CI); 3.98, (1.88-8.41)].

Even though calprotectin levels seemed to better estimate mortality at 90 days, we assessed whether the combination with CRP and NLR might improve mortality prediction. Univariate ROC curves for CRP and NLR rendered cut-off values of 7.1 mg/L and 3.32, respectively. When considering the combination of these three parameters, patients with calprotectin ≥ 2.26µg/mL, CRP ≥ 7.1 mg/L and NLR levels ≥3.32 had an estimated probability of dying in the next 90 days of 49%, whereas calculated mortality risk in patients with negative readings for all 3 markers was 3.7%. Further, we stratified our study population according to the presence of none, 1, 2 or 3 of these inflammatory markers above the cut-off values (Figure 3C). At 90 days, 1.6% of patients with negative readings had died. Also, 12.5% of patients with 1 positive marker, 30.7% of patients with 2 positive markers, and 42.3% of patients with 3 positive markers died.

[Insert Figure 3]
Predictors of 3-month functional independence

Variables associated with functional outcome in the univariate analysis were age, admission NIHSS score, intravenous tPA treatment, endovascular treatment, ASPECTS and ICH, as well as serum glucose, NLR, CRP and calprotectin values (Table 3).

In the multivariate analysis, calprotectin and CRP were not associated with 3-month FI, whereas NLR remained associated after adjusting for potential confounders [per a log+1 increase: OR, (95%CI); 0.53, (0.36-0.77); p=0.001]. Similar results were obtained, when the analysis was repeated evaluating the strongest predictors by stepwise selection (Table 3). Likewise, NLR was the only inflammatory marker which remained significantly associated with 3-month FI [0.52, (0.34-0.80); p=0.003]. Univariate ROC analysis for 3-month FI showed a NLR cut-off point for FI of 2.71 [AUC, (95%CI): 0.65, (0.58-0.71)]. When stratifying for this optimal cut-off and controlling for age, serum glucose, NIHSS score, and ICH, patients with NLR < 2.71 were 2.12 times more likely to be functional dependent at 90 days than AIS patients with an NLR ≥ 2.71 [OR, (95%CI); 2.12 (1.19-3.79)].

Table 3. Univariate and multivariate logistic regression models of 3-month functional independence with calprotectin and other baseline characteristics.
| Variable                                   | Univariate Logistic Regression | Multivariate Logistic Regression (n=324) |
|-------------------------------------------|-------------------------------|----------------------------------------|
|                                           | OR (95%CI)                     | p value                                | OR (95%CI)                     | p value                                |
| Age (per 10-year increase)                | 0.57 (0.46-0.71)              | <0.001                                 | 0.58 (0.44-0.76)              | <0.001                                 |
| Sex (female)                              | 0.67 (0.43-1.06)              | 0.089                                  |                              |                                        |
| Hypertension                              | 0.74 (0.45-1.21)              | 0.232                                  |                              |                                        |
| Type-2 Diabetes                           | 0.87 (0.53-1.42)              | 0.572                                  |                              |                                        |
| Dyslipidemia                              | 0.96 (0.61-1.50)              | 0.852                                  |                              |                                        |
| SBP at admission *                        | 1.40 (0.36-5.36)              | 0.628                                  |                              |                                        |
| (per 10mmHg increase)                     |                               |                                        |                              |                                        |
| DBP at admission                          | 1.01 (0.89-1.16)              | 0.830                                  |                              |                                        |
| (per 10mmHg increase)                     |                               |                                        |                              |                                        |
| Serum Glucose at admission (mg/dL) *      | 0.34 (0.16-0.71)              | 0.004                                  | 0.25 (0.10-0.64)              | 0.004                                  |
| Neutrophil count (x mm3) *                | 0.37 (0.20-0.66)              | 0.001                                  | ‡                            |                                        |
| Lymphocyte count (x mm3) *                | 2.40 (1.50-3.86)              | <0.001                                 | ‡                            |                                        |
| NLR *                                     | 0.44 (0.31-0.62)              | <0.001                                 | 0.52 (0.34-0.80)              | 0.003                                  |
| C-Reactive Protein (mg/L) *               | 0.64 (0.53-0.78)              | <0.001                                 |                              |                                        |
| Calprotectin µg/mL *                      | 0.49 (0.34-0.70)              | <0.001                                 |                              |                                        |
| NIHSS score at admission                  |                               |                                        |                              |                                        |
| 7-14 (vs 0-7)                             | 0.16 (0.08-0.31)              | <0.001                                 | 0.14 (0.07-0.31)              | <0.001                                 |
| >14 (vs 0-7)                              | 0.12 (0.07-0.21)              | <0.001                                 | 0.16 (0.08-0.32)              | <0.001                                 |
| ASPECTS at baseline ≥7                    | 18.95 (2.37-151.57)           | 0.006                                  |                              |                                        |
| Intravenous thrombolysis                  | 0.47 (0.30-0.74)              | 0.001                                  |                              |                                        |
Predictors of intracranial hemorrhage after ischemic stroke

Development of an ICH after AIS was associated with admission NIHSS score, intravenous tPA treatment and endovascular treatment, along with calprotectin and CRP in univariate analyses. Detailed results are presented in Additional file 2: Supplementary Table 1. Furthermore, ICH was more frequent in females. However, in multivariate analyses only sex, NIHSS score $\geq 14$ and intravenous tPA treatment remains statistically significant, and none inflammatory markers were independently associated with ICH after AIS.

Presence of S100A9 in thrombi retrieved from stroke patients

S100A9 protein was present in all thrombi analysed and quantification of thrombus constituents revealed that stroke thrombi contained on average 12.8% (IQR:1.34-28.89) red blood cells, 15.05% (4.84-26.65) platelets, 0.62% (0.35-1.17) leukocytes, and 3.52% (1.17-6.68) S100A9. Distribution pattern of S100A9 through the thrombus seemed to be related with leukocyte distribution and was primarily found at the interface between red blood cell-rich and platelet-rich areas. Besides the described distribution, S100A9 was also present within platelets islets (Figure 4A-B).

Interestingly, a positive correlation was observed between S100A9 and platelets (Pearson r 0.46, p<0.002); leukocytes (0.45, p<0.01) and neutrophil elastase (0.70, p<0.001) thrombi content. When evaluating the amount of S100A9 in thrombi and its association with calprotectin circulating levels, we did not see a correlation. Furthermore, no correlation between thrombus S100A9 content and age, sex, functional outcome and stroke severity was observed (not shown).
Finally, we observed a tendency to higher thrombi S100A9 amount in cardioembolic thrombi and in those who had died (Figure 4C-D). The small sample size of atherothrombotic thrombi and the low number of deaths did not allow us to reach statistical significance (Additional file 2: Supplementary Table 2).

**Discussion**

Our study shows that plasma calprotectin is a strong independent inflammatory predictor of 3-month mortality in AIS, and that improves the risk prediction of all-cause death associated to well-established factors as stroke severity, high blood glucose or baseline mRS. Furthermore, S100A9 as part of calprotectin heterodimer was present in all thrombus obtained by MT in stroke patients and seems to be higher in thrombi from patients who died and in those of cardioembolic etiology. Moreover, NLR was the only inflammatory marker that remained significantly associated with 3-month FI whereas CRP and calprotectin did not. None of these three inflammatory biomarkers were independently associated with ICH after AIS.

Increasing evidence shows that inflammatory response plays an important role in various processes related to AIS [3]. Brain ischemia causes an immediate local immuno-inflammatory reaction with strong activation of microglia, astrocytes and endothelial cells; and release of cytokines both from activated cells and endothelium [18]. This non-specific response after brain tissue damage results in blood-brain barrier permeability and infiltration of inflammatory cells into ischemic area [19] which have the potential to further increase tissue injury.

Calprotectin is produced by neutrophils and infiltrating macrophages in ischemic brain [20]. Recently, in an experimental model of transient focal ischemia, inhibition of S100A9 reduced infarct volume and brain swelling [20] and a therapeutic vaccine against MRP14 (S100A9) resulted in thrombosis inhibition in a murine model of ischemic stroke [12]. In addition, calprotectin overexpression has been localized in ischemic hemisphere CD11b-positive cells [20] and brain proteomic analysis has shown that calprotectin was up-regulated in experimental models of brain ischemia [21]. Upon cell activation, calprotectin is released and can be detected in serum or body fluids as a clinical inflammatory marker. In plasma, it is a relatively stable and easily measurable protein, and has been proposed as a potential biomarker of inflammatory processes. Calprotectin was previously shown as a useful biomarker of cardiovascular disease risk [9] and cardiovascular event and recurrence [22]. A very recent study of 4785 patients with AIS from 2 independent cohorts has reported that high plasma calprotectin concentrations at baseline were independently associated with poor prognosis and dead within 3 months after ischemic stroke [23]. Our study confirms these results and extends previous data showing that higher calprotectin levels are associated with 3-month mortality in AIS patients with greater predictive power for mortality than other studied inflammatory markers.

CRP and NLR have been shown as potential biomarkers or therapeutic targets for stroke management [3,24]. CRP is a sensitive indicator of inflammation [6] widely used in clinical practice. In a case-control study of 600 AIS patients, CRP levels were significantly higher for all ischaemic subtypes than controls,
both in the acute phase and at 3-month follow-up examinations [7]. Moreover, it has been reported that CRP was independent predictors of short-term outcome and mortality after AIS [25]. Likewise, higher NLR values have been shown as independent predictors of ICH and 3-month mortality [4,5] in patients with large vessel occlusion who underwent a mechanical thrombectomy. Besides, an early increase of neutrophils in patients with AIS has been associated with larger infarct volumes [26]. In agreement with these data, CRP and NLR were independently associated with 3-month mortality in our study and NLR was independently associated with FI in AIS patients acting as the strongest predictor for FI among the inflammatory markers tested. However, the better prediction power of calprotectin for poor prognosis and mortality, as well as its association with other inflammatory cells and proteins, suggest that it could be a useful biomarker of immune-neutrophilic activation after AIS associated with worse outcomes.

In line with previous reports suggesting a multimarker approach to improve outcome prediction in AIS patients [27], our data indicate that calprotectin either alone or even better when combined with CRP and NLR could improve the overall prediction of 3-month mortality in AIS, suggesting that an elevated inflammatory state may contribute to the mortality in AIS patients. Though, the predictive capacity of this multimarker approach in our cohort and the robust results on K-fold internal cross-validation, it will need further external validation in other cohorts of AIS patients.

Because little was known about the composition of human stroke thrombi, we assessed for the first time the presence of S100A9 in thrombi retrieved from stroke patients. S100A9 forms a heterodimer with S100A8 (Calprotectin), and has been reported as a key molecule in the regulation of thrombus formation [13]. It is expressed and secreted in blood by platelets and neutrophils after vascular injury [28]. Moreover, S100A9 gene knockout or neutralization reduces neutrophil recruitment and thrombotic effects by modulating platelet function without influencing other haemostatic parameters [12]. S100A9 is expressed in carotid [28] and femoral atherosclerotic plaques [10], and in human coronary artery thrombus [13]. Our histological analysis showed that S100A9 is present in all ischemic stroke thrombi and correlated with inflammatory cells and platelets. According to our results, thrombus with confirmed cardiac source and from patients who died during 3-month follow-up seem to have higher amount of S100A9 whereas no statistically significant differences were achieved. Further studies are needed to elucidate whether S100A9 content or organization in stroke thrombi is associated with thrombus formation, and resolution.

There are some limitations to this report. First, the modest sample size and the retrospective analysis of prospectively collected data are important methodological shortcomings. Second, the possibility of an unknown confounding factor cannot be ruled out completely. Moreover, calprotectin was measured in blood samples obtained at different times from onset for each patient (although always within 24h after stroke onset). Finally, the observational study design did not allow establishing a cause-effect relationship.

**Conclusions**
This study expands the existing evidence by showing that plasma calprotectin is independently related to 3-month mortality and could be used in clinical practice as a novel prognostic biomarker, providing complementary information to other inflammatory biomarkers in AIS patients. Further studies are needed to determine its influence in thrombus formation and resistance to reperfusion.

**Abbreviations**

AIS: acute ischemic stroke; ICH: intracranial haemorrhage; FI: functional independence; mRS: modified Rankin Scale; NLR: Neutrophil-Leukocyte ratio; CRP: C-reactive Protein; MRP8/14: myeloid-related protein -8/-14; SBP: systolic blood pressure; DBP: diastolic blood pressure; NIHSS: National Institute of Health Stroke Scale; tPA: tissue plasminogen activator; LDL: Low-density lipoprotein; TOAST: Trial of Org 10172 in Acute Stroke Treatment; ASPECTS: Alberta Stroke Program Early CT Score; MSB: Martius Scarlet Blue staining; SD: Standard Deviation; IQR: interquartile range; OR: Odds Ratio; ROC: Receiver-Operating Characteristics.

**Declarations**

**Ethics approval and consent to participate:** Written informed consent was obtained from all study participants. The study was performed according to the guidelines of the Helsinki Declaration and was approved by the Ethics Committee of Gobierno de Navarra (ref.2015/21).

**Consent for publication:** Not applicable.

**Availability of data and materials:** The data that support the findings of this study are available from the corresponding author on reasonable request.

**Competing interest:** The authors declare no potential conflicts of interests with respect to the research, authorship or publication of this manuscript.

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**Author Contribution:** JM, RM and JO conceived the study. JM, IR, MH, NA, SM, BZ and RM collected samples and recruited patients. MN, JR, JP, MM and JO processed samples and developed the experiments. JM, MN and JO wrote the paper. All the authors have reviewed the manuscript before submitting.

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Figures
Figure 1

Histological staining and quantification of stroke thrombi. A, Representative adjacent thrombus slides stained with Martius Scarlet Blue (MSB, left superior) and immunostained for platelets (CD42b), S100A9 and neutrophil elastase (in brown). B, Magnifications of the area selected in panel A. MSB staining shows a red blood-rich area (orange-yellow, arrowhead) near to a collagen area (light blue, arrow). A fibrin area at the periphery appears in red (asterisk). Platelet-rich regions (CD42b staining in brown) are related with collagen areas. Neutrophil elastase (brown) tends to accumulate at the boundary of platelet-rich zones (brown with CD42b staining) and S100A9 distribution seems to be related with neutrophils and monocytes (neutrophil elastase +) presence at the interface between red blood cell-rich and platelet-rich areas and also within platelets islets. C, Box-plot showing the differences in percentage of S100A9 staining in thrombi retrieved from cardioembolic and atherothrombotic stroke patients. Calculated by Wilcoxon ranksum test. D, Box-plot showing the differences of S100A9 percentage in thrombi from patients who died or survived after stroke. Calculated by Student t test over log-transformed S100A9.
Figure 2

Prognostic value of stroke severity and inflammatory biomarkers to predict 3-month mortality. A, ROC analysis of univariate regression models for 3-month mortality with NIHSS (blue), calprotectin (red), CRP (green) and NLR (yellow). B, ROC curves of multivariate model of 3-month mortality with (red) and without calprotectin (blue). C, 3-month mortality rates in our population stratifying according to the presence of 1, 2 or 3 inflammatory markers above the cut-off values. NIHSS, National Institute of Health Stroke Scale; CRP, C-reactive protein; NLR, neutrophil-lymphocyte ratio.
Figure 3

Box-plots of plasma calprotectin levels by outcomes. Differences in: A. 3-month mortality; B. ICH after AIS; C. 3-month FI. ICH, intracranial haemorrhage; AIS, acute ischemic stroke; mRS; modified Rankin Scale. Calculated by Wilcoxon ranksum test.
Patients with suspected AIS attended in our Stroke Unit during the study period (N=748)

Suspected AIS (n=511):

Excluded (n=237):
- Transient ischemic attack (187)
- Stroke mimics (50)

Suspected AIS (n=511):

Excluded (n=44):
- No acute ischemia in neuroimage (44)

AIS with established ischemia (n=467)

Excluded (n=54):
- Systemic inflammation or infection at onset (54).

Total included (n=413)

Figure 4

Flowchart of the study.

Supplementary Files

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- Additionalfile1.pptx
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