Received: 26 October 2022 | Revised: 30 March 2023 | Accepted: 13 April 2023

https://doi.org/10.1016/j.jtha.2023.04.015

ORIGINAL ARTICLE

The thrombus proteome in stroke reveals a key role of the innate immune system and new insights associated with its etiology, severity, and prognosis

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Abstract

Background: Nowadays little is known about the molecular profile of the occluding thrombus of patients with ischemic stroke.

Objectives: To analyze the proteomic profile of thrombi in patients who experienced an ischemic stroke in order to gain insights into disease pathogenesis.

Methods: Thrombi from an exploratory cohort of patients who experienced a stroke were obtained by thrombectomy and analyzed by sequential window acquisition of all theoretical spectra-mass spectrometry. Unsupervised k-means clustering analysis was performed to stratify patients who experienced a stroke. The proteomic profile was associated with both the neurological function (National Institute of Health Stroke Scale [NIHSS]) and the cerebral involvement (Alberta Stroke Program Early CT Score [ASPECTS]) prior to thrombectomy and the clinical status of patients at 3 months using the modified Rankin Scale. In an independent cohort of 210 patients who experienced a stroke, the potential role of neutrophils in stroke severity was interrogated.

Results: Proteomic analysis identified 580 proteins in thrombi, which were stratified into 4 groups: hemostasis, proteasome and neurological diseases, structural proteins, and innate immune system and neutrophils. The thrombus proteome identified 3 clusters of patients with distinctive severity, prognosis, and etiology of the stroke. A protein signature clearly distinguished atherothrombotic and cardioembolic strokes. Several proteins were significantly correlated with the severity of the stroke (NIHSS and ASPECTS). Functional proteomic analysis highlighted the prominent role of neutrophils in stroke severity. This was in line with the association of neutrophil activation

Manuscript handled by: Karl Desch

Final decision: Karl Desch, 14 April 2023

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Funding information
RYC2021-033828-I, funded by Ministerio de Ciencia e Innovación/agencia estatal de investigación/10.13039/501100011033 and the European Union "NextGenerationEU"/PRTR. RyC-2017-23437.

markers and count with NIHSS, ASPECTS, and the modified Rankin Scale score 90 days after the event.

Conclusion: The use of sequential window acquisition of all theoretical spectra-mass spectrometry in thrombi from patients who experienced an ischemic stroke has provided new insights into pathways and players involved in its etiology, severity, and prognosis. The prominent role of the innate immune system identified might pave the way for the development of new biomarkers and therapeutic approaches in this disease.

KEYWORDS
biomarkers, immune system, proteomics, stroke, thrombi

1 | INTRODUCTION

Stroke is considered one of the primary causes of mortality and disability worldwide. Due to aging of the population, the prevalence of stroke is expected to increase in the next years, along with the annual cost associated with treatments, diagnostic tests, health care procedures, etc. [1].

Approximately 80% to 85% of all strokes are ischemic strokes, mainly led by arterial occlusion from causes such as atrial fibrillation or valvular heart disease (cardioembolic) or from atherosclerotic disease in the main intracranial and extracranial cerebral arteries (atherothrombotic) [2].

Recent advances have increased knowledge of the biological mechanisms involved in the pathogenesis of stroke, highlighting the role of several processes, such as necrosis, inflammation, oxidative stress, complement activation, impairment of the blood-brain barrier, and loss of homeostasis, among others [3]. Nonetheless, there is still a lack of understanding of the full mechanism in which all of these and other processes are interconnected to drive the development and progression of stroke.

Despite recent efforts to identify several biomarkers that can help improve diagnosis, prognosis and therapeutic stratification, and monitoring, no individual markers have demonstrated to have an optimum performance to be implemented in the acute clinical setting [4]. The different types of samples evaluated, the timing of biomarker measurement, and the validation process in independent cohorts constitute important factors that need to be taken in consideration to achieve this challenging goal [5].

In this sense, the advent of endovascular thrombectomy, which improves stroke outcomes, represents an unprecedented opportunity to obtain a valuable and unique sample from the vascular bed where cerebral damage is happening, which might reveal relevant findings [6].

Overall, the integration of high-throughput technologies and advanced computational tools to profile unique samples, such as thrombi, might represent a more realistic approach to identify new and more specific biomarkers and gain insight into the pathophysiology of the disease [7].

Proteins are the most important class of biomolecules in the context of understanding real-time human biology. They are the executors of most biological processes (eg, transcription factors, enzymes, and cytokines) and dynamic indicators of health and disease phenotypes. In addition, proteins are the targets of most current drugs as they provide the most actionable targets for therapeutic interventions [8].

In this study, we characterized for the first time the protein composition of thrombi from a cohort of patients who experienced acute ischemic stroke using sequential window acquisition of all theoretical spectra-mass spectrometry (SWATH-MS), a high-throughput mass spectrometry technique [9,10], in combination with a prospective clinical assessment using bioinformatic tools. Our results try to reveal new biological pathways involved in the etiology, severity, and progression of the stroke, highlighting the key role of the innate immune system in those processes.

2 | METHODS

2.1 | Patients, clinical data, and samples

Thrombi from a cohort of 18 consecutive patients who experienced acute ischemic stroke were collected following endovascular thrombectomy at Reina Sofia University Hospital of Córdoba (Spain) within a timeframe of 6 months. Patients provided written informed consent. The study was conducted in accordance with the Declaration of Helsinki principles.

A thorough clinical and prospective evaluation was performed, including demographic variables (sex and age), cardiovascular risk factors (hypertension, dyslipidemia, obesity, diabetes, atrial fibrillation, smoking, and drinking habits), comorbidities (ischemic heart disease, transient ischemic attack, previous stroke, and chronic obstructive pulmonary disease), and etiology of the stroke (cardioembolic or

atherothrombotic). Moreover, different scores of severity and prognosis of patients who experienced stroke were also obtained, such as the Alberta Stroke Program Early CT Score (ASPECTS), which evaluates early ischemia signs on computed tomography; the National Institute of Health Stroke Scale (NIHSS), which assesses the stroke severity; and the modified Rankin Scale (mRS), which determines the degree of disability (Table).

The prognosis of the stroke suffered by these patients was evaluated with the mRS score 90 days after the event (mRS90).

Fibrinolytic agents were administered in 8 patients before the thrombectomy, whereas antiplatelet and antithrombotic agents were administered in 2 and 5 patients, respectively, before the stroke.

The sites of occlusion from which the clots were retrieved included complete internal carotid artery (9 patients), proximal medial cerebral artery (3 patients), terminal internal carotid artery (2 patients), distal medial cerebral artery (2 patients), intracranial internal carotid artery (1 patient), and posterior cerebral artery (1 patient).

In an independent cohort, clinical data on 210 patients suffering acute ischemic stroke from both the Reina Sofia University Hospital of Córdoba (Spain) and the Navarre University Hospital (Spain) were retrospectively collected to evaluate the association between immune cells (neutrophil count) and the severity and prognosis of stroke using the different scores (ASPECT, NIHSS, and mRS90) (Supplementary Table S1).

TABLE Clinical characteristic of patients who experienced a stroke (N = 18).

Clinical data	Mean/%
Age (y), mean ± SD	63.1 ± 15.9
Sex, female/male (%)	44.8/55.2
Cardioembolic/atherothrombotic event (%)	50/50
Hypertension (%)	63.8
Dyslipidemia (%)	51.7
Obesity (%)	32.8
Type II diabetes (%)	20.7
Atrial fibrillation (%)	31.0
Smoking habit (%)	37.9
Drinking habit (%)	17.2
Ischemic heart disease (%)	13.8
Previous stroke/TIA (%)	13.8
Previous COPD (%)	6.9
Wake-up stroke (%)	14.5 %
ASPECTS, mean ± SD	7.2 ± 1.6
NIHSS, mean ± SD	14.5 ± 5.8
mRS, mean ± SD	0.2 ± 0.5

ASPECTS, Alberta Stroke Program Early CT Score; COPD, chronic obstructive pulmonary disease; mRS, modified Rankin Scale; NIHSS, National Institute of Health Stroke Scale; TIA, transient ischemic attack.

In addition, neutrophils from 50 consecutive patients from the Reina Sofia University Hospital of Córdoba (Spain) included in the independent cohort were purified through immunomagnetic selection using autoMACS (Miltenyi Biotec) to evaluate the association between activation markers and the severity and progression of the stroke.

2.2 | SWATH-MS

2.2.1 | Sample preparation

Thrombi obtained by endovascular thrombectomy (through ≥1 passes for complete recanalization) were processed individually. In case >1 fragment was obtained, they were pooled. Clots were washed twice in phosphate-buffered saline and frozen in liquid nitrogen until further processing. Then, they were washed again in phosphate-buffered saline, and the tissues were mechanically disrupted using an extraction buffer.

Finally, the samples were centrifuged to remove cell debris, and 50 μg of proteins from the supernatant were digested with a protocol described previously [11].

To extract the proteins, a cell lysis solution was used, including the following reagents and concentrations: urea (7M), thiourea (2M), Tris buffer (30 mM), and CHAPS buffer (4%) (all from Sigma-Aldrich–Merck). Trypsin was used as a protease to digest peptides (sequencing grade modified trypsin, Promega).

Representative photographs from clots are displayed in Supplementary Figure S1.

2.2.2 | Mass spectrometry

For all liquid chromatography–mass spectrometry (LC-MS) analysis, data-dependent acquisition (DDA), and Data-Independent Adquisition SWATH (DIA-SWATH), 1 μg of total digested samples were injected in a hybrid quadrupole time-of-flight (Q-TOF) mass spectrometer (Triple TOF 5600+, Sciex) coupled online to nano-high-performance liquid chromatography (HPLC) (Ekspert nLC415, Eksigent). For higher sensitivity, both DDA and sequential window acquisition of all theoretical spectra (SWATH) runs were performed at nano-flow (300 nL/min) in a 25-cm long × 75-μm internal diameter column (Acclaim PepMap 100, Thermo Scientific) using a 120-minute gradient from 5% to 30% B (A: 0.1% formic acid [FA] in water; B: 0.1% in acetonitrile [ACN]).

2.2.3 | Data analysis

The MS analysis was divided into the following steps. i) Building of an tandem mass spectrometry (MS/MS) peptide library from the peptides and proteins identified from DDA shotgun nano–liquid chromatography (LC)-MS/MS runs obtained from pools made from all samples. This step was performed by combining the use of Protein Pilot software (v5.0.1, Sciex) with a human Swiss-Prot protein

database (including RePliCal iRT peptides [PolyQuant GmbH]) and PeakView software (v.2.1, Sciex). ii) Analysis of each sample by DIA-SWATH-MS nano–LC-MS/MS runs with a variable SWATH LC-MS method: full time-of-flight mass spectrometry (TOF-MS) scan (350-1250 m/z, acquisition time; 50 milliseconds) followed by 50 windows of variable size (230-1500 m/z; an acquisition time of 90 milliseconds) with a minimum size of 5 m/z and a window overlap of 1 m/z). (iii) Analysis of protein identification and quantification data for the proteins contained in the DDA spectral library previously obtained by extraction of fragment ion chromatograms using the mass spectometry/mass spectrometry all with SWATH Acquisition MicroApp (v.2.0, Sciex) and peptide retention times calibration in all runs using spiked RePliCal iRT peptides (also present in the library).

Additionally, to be as confident as possible with identifications and quantifications, only scores of >99% and a false discovery rate of <1% were included in the analysis. Following this step, MarkerView (v.1.2.1, Sciex) was used to normalize all acquired data, and a *t*-test was performed for testing differential abundance. Only proteins detected in all the samples (580) were finally selected for subsequent analyses. Proteins with missing values in any samples were not included.

All mass spectrometry and identification and quantification analysis were performed at IMIBIC Mass Spectrometry and Molecular Imaging Unit.

The data are available in the public scientific repository Figshare [12] under the accession number 22269277 and https://doi.org/10.6084/m9.figshare.22269277. See Supplementary Methods for further details.

2.3 | Neutrophil activity markers

Several neutrophil markers, including *CD66B*, *IL8*, *PDE4B*, *SOD2*, *CCT2*, *CCT8*, *CD47*, and *KPNB1*, were analyzed in neutrophils purified from patients who experienced stroke by real-time polymerase chain reaction (PCR). Briefly, RNA was extracted using an RNA/DNA/protein purification kit (Norgen Biotek Corp) following the manufacturer's recommendations. RNA purity was verified through the optical density (OD) absorption ratio OD260/OD280 between 1.8 and 2.0 with nanodrop. For first strand complementary DNA synthesis, 0.5 μg of total RNA was reverse transcribed using the commercial kit PrimeScript RT Master Mix (TAKARA Bio) following the manufacturer's instructions. Gene expression was assessed by real-time PCR using a LightCycler Thermal Cycler System (Roche Diagnostics). The reaction was performed following the manufacturer's protocol with GoTaq qPCR Master Mix from Promega and consisted of initial denaturation of 10 minutes at 95 °C, then 40 cycles of 15-second denaturation at 95 °C, and 1 minute of annealing and extension phase at 60 °C. A threshold cycle (Ct value) was obtained for each amplification curve, and a ΔCt value was first calculated by subtracting the Ct value for human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) complementary DNA from the Ct value for each sample and transcript. Fold changes compared with the endogenous control were then determined by calculating $2^{-\Delta Ct}$. Every sample was performed in triplicate and negative controls were included in all the reactions. A list of primer sequences (Sigma-Aldrich) used in this study is displayed in Supplementary Table S2.

In parallel, circulating neutrophil elastase was also evaluated in the serum of patients who experienced a stroke as a marker of NETosis using the Human PMN Elastase ELISA kit (Abcam), following the manufacturer's instruction.

2.4 | Bioinformatic and statistical analyses

Interactions among all the 580 proteins identified in thrombi from patients who experienced a stroke through the SWATH-MS analysis were queried and analyzed through the STRING (https://string-db.org) platform, which identified main enriched pathological pathways and networks of known and predicted protein–protein interactions. Moreover, Gene Ontology enrichment analysis was performed to identify biological pathways significantly enriched and associated with each network.

Unsupervised k-means clustering analyses were performed with the MetaboAnalyst platform [13] to identify subgroups of patients on the basis of specific proteomic profiles.

Statistical analysis and graphical representation of results were performed using GraphPad Prism 8 software. The normal distribution of the variables to characterize the differences in the analyzed parameters was assessed using the Kolmogorov-Smirnov test.

Based on this test, comparisons between quantitative and qualitative variables were made using the Student's *t*-test or, alternatively, using a nonparametric test (Mann–Whitney U-test).

Correlations were assessed by Spearman's rank correlation. Differences were considered significant at $p < .05$.

3 | RESULTS

3.1 | Proteomic characterization of stroke thrombus

The SWATH-MS analysis of thrombi from patients who experienced a stroke identified 580 proteins in all the evaluated samples (Supplementary Table S3). In order to interrogate the biological meaning of the proteomic composition of the thrombus, these proteins were functionally classified into 4 different molecular clusters.

As expected, a cluster of proteins associated with the coagulation process was identified, where biological pathways and functions such as hemostasis, platelet degranulation, activation, signaling and aggregation, and fibrin clot formation were the most significantly enriched. In terms of expression, individual proteins such as hemoglobin, fibrinogen, coagulation factor XIII A, spectrin, thrombospondin-1, and platelet glycoprotein, among others, were the most abundant (Figure 1A).

Another important cluster integrated by structural proteins was identified, where biological pathways such as regulation of actin

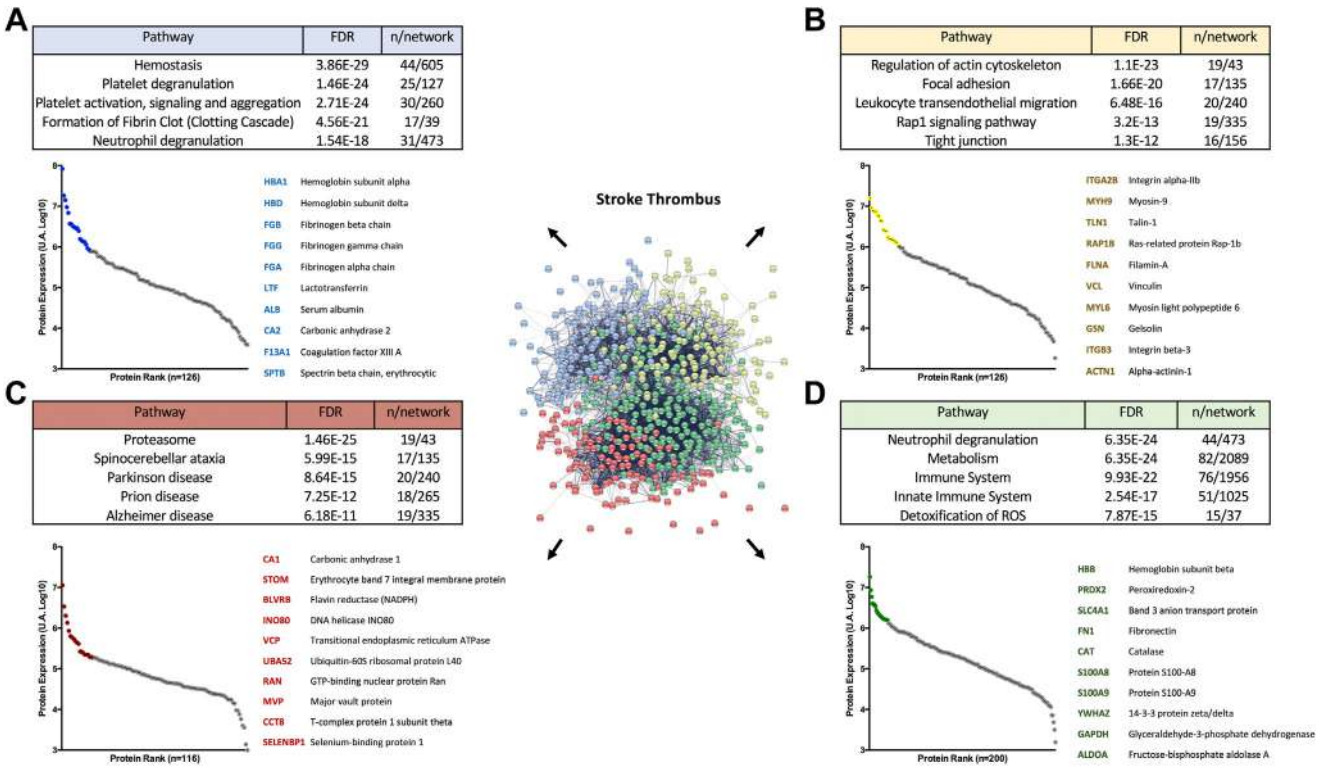


FIGURE 1 Proteomic profile of stroke thrombus. Sequential window acquisition of all theoretical spectra-mass spectrometry analysis of protein content in thrombus from patients with stroke obtained by thrombectomy. (A–D) Clusters of proteins associated with distinctive biological pathways and functions. Tables represent functional enrichments where the false discovery rate (FDR) and the number of proteins in each pathway are displayed. Lists of the proteins more expressed in each cluster are also represented.

cytoskeleton, focal adhesion, leukocyte migration, Rap1 signaling, and tight junction were enriched. Relevant proteins involved in those processes, including integrins, myosins, talin, filamin, actinin, vinculin, gelsolin, and tubulin, among others, were highly expressed in the thrombus of patients who experienced stroke (Figure 1B).

A third cluster of proteins related to the proteasome and neurologic diseases, such as Spinocerebellar ataxia, Parkinson disease, Alzheimer disease, and Prion disease, were also characterized. The most abundant proteins included in this cluster were enzymes mainly associated with metabolic processes, such as carbonic anhydrase, nicotinamide adenine dinucleotide phosphate, DNA helicase, ATPase, glycosyltransferase, and ubiquitin proteins (Figure 1C).

Finally, an interesting cluster of proteins associated with the innate immune system, where the neutrophils seem to have a prominent role, was also noticed. In fact, pathways associated with the biology of these cell types, including degranulation, immune response, metabolism, and oxidative stress, were significantly enriched. Individual proteins involved in those processes, such as peroxiredoxins, catalase, glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase A, pyruvate kinase, and histone H2B, among others, were highly expressed in this cluster (Figure 1D).

Besides, the levels of the different proteins were highly correlated in the thrombus, suggesting a coordinated process underlying the clot formation (Supplementary Figure S2).

3.2 | Unsupervised clustering analysis of the thrombus proteome identified subgroups of patients with distinctive severity, prognosis, and etiology of the stroke

The identification of distinctive molecular phenotypes among all the patients who experienced a stroke profiled at the proteomic level in the thrombus was performed by an unsupervised clustering analysis. The analysis revealed 3 subgroups of patients with distinct levels of the proteomic signature previously identified in the thrombus (Figure 2A). The principal component analysis confirmed the presence of these 3 groups of patients clearly stratified according to the thrombi proteome.

Interestingly, the stratification of patients in different clusters based on the thrombus proteome was able to unravel important clinical features hidden among the patients evaluated. Thus, regarding the etiology of the thrombus, all the patients included in cluster 2 suffered an atherothrombotic stroke, whereas all the patients included in cluster 3 presented a cardioembolic stroke. Cluster 1 was characterized by 60% of patients who experienced a stroke with an atherothrombotic etiology and 40% with a cardioembolic etiology (Figure 2B).

The proteomic profile was directly associated with the number of passes performed during thrombectomy for complete recanalization.

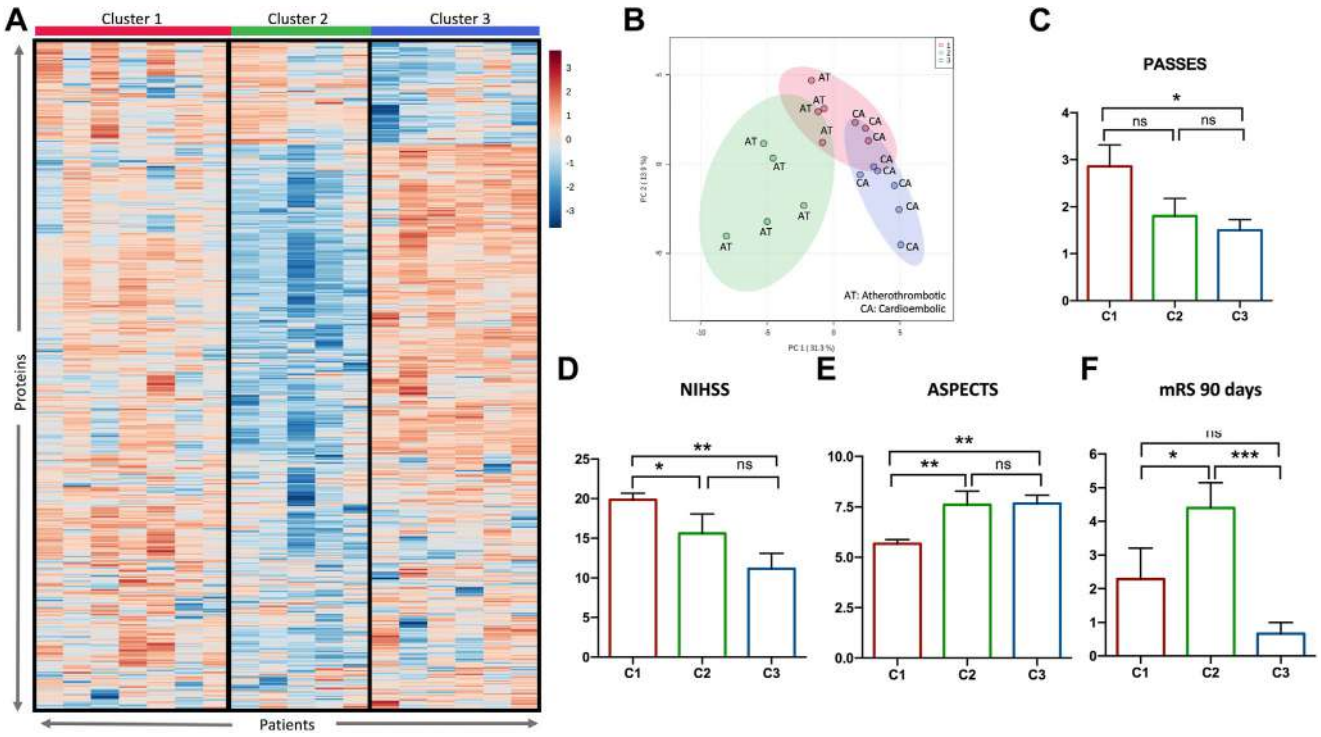


FIGURE 2 Clustering analysis of patients with stroke based on thrombus proteome and clinical association. (A) Heat map representing the levels of all the proteins identified in the thrombus through sequential window acquisition of all theoretical spectra-mass spectrometry assay. Three clusters of patients obtained by k-means unsupervised cluster analysis are also highlighted. Red and blue colors indicate high and low levels, respectively. (B) Principal component analysis showing the stratification of the patients in the 3 groups following the proteomic analysis of the thrombus. (C) Association of the proteomic clusters with the number of recanalization passes during thrombectomy. (D, E) Association of the different clusters of patients with the severity of the stroke at functional (National Institute of Health Stroke Scale [NIHSS]) and anatomical levels (Alberta Stroke Program Early CT Score [ASPECT]). (F) Association of the clusters of patients with stroke and the clinical outcome at 3 months determined with the modified Rankin Scale (mRS) score. ns, not statistically significant; PC1, principal component 1; PC2, principal component 2. $^{*}p < .05$. $^{**}p < .01$. $^{***}p < .001$.

Thus, patients belonging to cluster 3 displayed the lowest number of passes required, whereas patients from cluster 1 showed the highest number of passes (Figure 2C).

Furthermore, patients from cluster 1 exhibited a more severe stroke in relation to patients from clusters 2 and 3 at both functional and anatomical levels, evidenced by the NIHSS and ASPECTS scores, which were significantly higher and lower, respectively (Figure 2D, E).

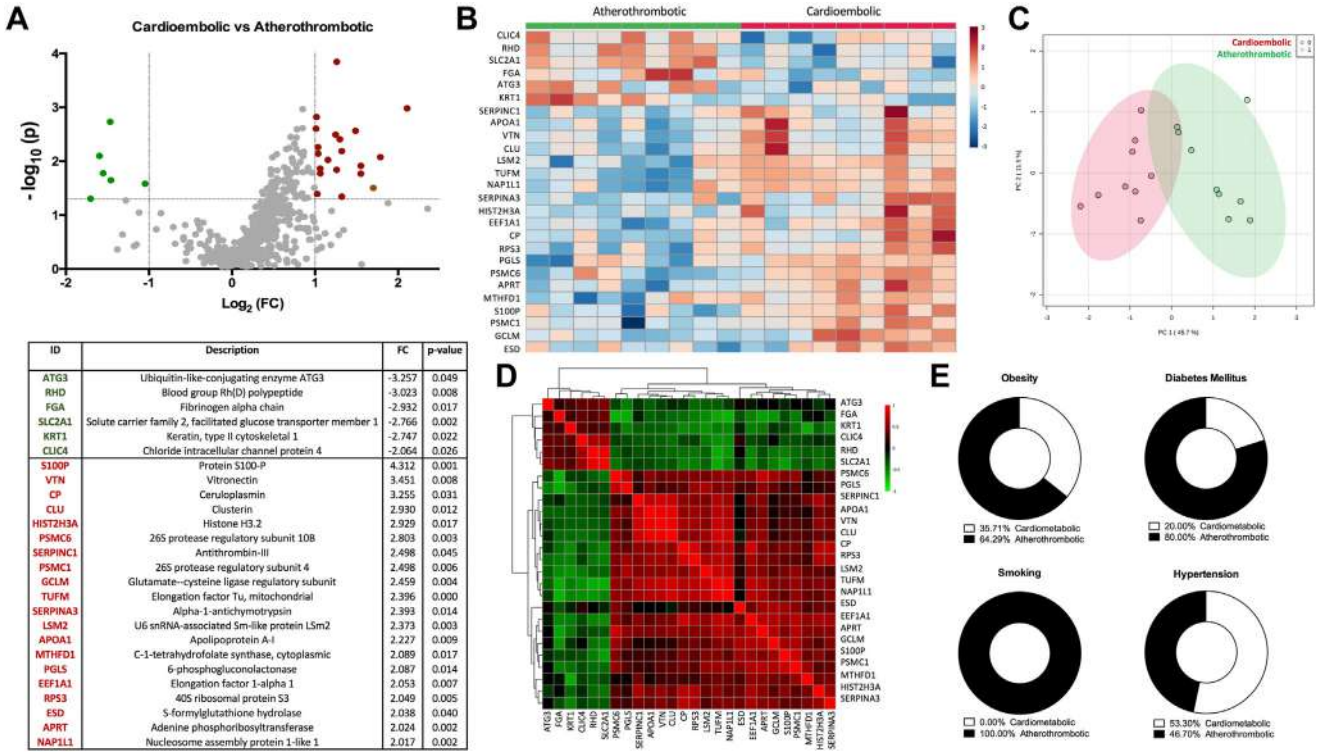
The distinctive proteomic profiles of the thrombus were associated not only with the severity and etiology of the stroke but also with the clinical patients' outcome. Patients from cluster 2 showed a worse clinical prognosis 3 months after the stroke, with an mRS90 score significantly higher than that of patients from clusters 1 and 3 (Figure 2F).

3.3 | The cardioembolic and atherothrombotic etiology of patients who experienced stroke is reflected in the differential protein content of their thrombi

Next, the identification of individual proteins more directly associated with the differential etiology of the thrombi of patients who experienced a stroke was performed by an analysis comparing the levels of all the proteins identified in the SWATH-MS assay between a thrombus of cardioembolic and atherothrombotic etiology.

Thus, 26 proteins were differentially expressed between cardioembolic and atherothrombotic thrombi. The levels of 6 proteins were significantly higher in atherothrombotic thrombi (lower in cardioembolic), including ATG3, RHD, FGA, SLC2A1, KRT1, and CLIC4, whereas the levels of 20 proteins were significantly higher in cardioembolic thrombi (lower in atherothrombotic), including S100P, VTN, CP, CLU, HIST2H3A, PSMC6, SERPINC1, PSMC1, GCLM, TUFM, SERPINA3, LSM2, APOA1, MTHFD1, PGLS, EEF1A1, RPS3, ESD, APRT, and NAP1L1 (Figure 3A). The main biological pathway enriched by this signature of proteins was the innate immune system (false discovery rate, 0.000879), highlighting the relevant influence of these cells in the mechanisms underlying the differential etiology of thrombus formation.

Furthermore, the signature integrated by these specific proteins allowed the unsupervised stratification of patients with stroke according to the cardioembolic or atherothrombotic etiology of the thrombus (Figure 3B, C). The distinctive pathologic processes associated with the generation of the atherothrombotic and cardioembolic



ID	Description	FC	p-value
ATG3	Ubiquitin-like-conjugating enzyme ATG3	-3.257	0.049
RHD	Blood group Rh(D) polypeptide	-3.023	0.008
FGA	Fibrinogen alpha chain	-2.932	0.017
SLC2A1	Solute carrier family 2, facilitated glucose transporter member 1	-2.766	0.002
KRT1	Keratin, type II cytoskeletal 1	-2.747	0.022
CLIC4	Chloride intracellular channel protein 4	-2.064	0.026
S100P	Protein S100-P	4.312	0.001
VTN	Vitronectin	3.451	0.008
CP	Ceruloplasmin	3.255	0.031
CLU	Clusterin	2.930	0.012
HIST2H3A	Histone H3.2	2.929	0.017
PSMC6	26S protease regulatory subunit 10B	2.803	0.003
SERPINC1	Antithrombin-III	2.498	0.045
PSMC1	26S protease regulatory subunit 4	2.498	0.006
GCLM	Glutamate--cysteine ligase regulatory subunit	2.459	0.004
TUFM	Elongation factor Tu, mitochondrial	2.396	0.000
SERPINA3	Alpha-1-antichymotrypsin	2.393	0.014
LSM2	U6 snRNA-associated Sm-like protein LSm2	2.373	0.003
APOA1	Apolipoprotein A-I	2.227	0.009
MTHFD1	C-1-tetrahydrofolate synthase, cytoplasmic	2.089	0.017
PGLS	6-phosphogluconolactonase	2.087	0.014
EEF1A1	Elongation factor 1-alpha 1	2.053	0.007
RPS3	40S ribosomal protein S3	2.049	0.005
ESD	S-formylglutathione hydrolase	2.038	0.040
APRT	Adenine phosphoribosyltransferase	2.024	0.002
NAP1L1	Nucleosome assembly protein 1-like 1	2.017	0.002

FIGURE 3 Differential protein profile between cardioembolic and atherothrombotic thrombus from patients with stroke. (A) Volcano plot (above) comparing differential protein levels between thrombus of cardioembolic and atherothrombotic etiology. A list of the differential proteins along with fold change (FC) and *p* value is displayed. Green color indicates higher expression in atherothrombotic thrombus, and red color in cardioembolic. (B) Heatmap showing individual levels of the differential protein signature that stratify patients according to the thrombus etiology. Red and blue colors indicate high and low expression, respectively. (C) Principal component analysis confirming the discrimination between the 2 types of thrombi using the differential protein signature. (D) Heat map of correlation between the differential protein signatures. Red and green color indicates positive and negative correlations, respectively. Color intensity represents the Pearson correlation coefficient. (E) Clinical features associated with the thrombus of cardioembolic and atherothrombotic etiology. PC1, principal component 1; PC2, principal component 2.

thrombi seemed to be highly coordinated, as suggested by the strong correlation found among all the proteins present in this signature (Figure 3D).

Along with the differential protein content, several clinical features were distinctively associated with the etiology of the thrombus. Thus, patients who suffered an atherothrombotic stroke were characterized by a higher proportion of obesity (65%), diabetes mellitus (80%), and smokers (100%) compared with patients who suffered a stroke with a cardioembolic etiology. No differences were observed among patients with hypertension (Figure 3E).

3.4 | The levels of specific proteins in the thrombus correlate with the severity of the stroke

Last, correlations analyses were performed to identify key proteins or pathways directly associated with the severity of the stroke among all the proteins identified in the SWATH-MS assay between 2 different severity scores such as NIHSS (increase with severity) and ASPECTS (decrease with severity).

The levels of 50 proteins were significantly correlated with the NIHSS score, where high levels of 27 proteins and low levels of 23 proteins were associated with a more severe stroke in terms of impairment of brain function (Figure 4A). The analysis of the functional involvement of these showed that the proteins that positively correlated with the severity of the disease were interconnected in a network enriched in biological pathways, such as the innate immune system, protein ubiquitination, and metabolism (Figure 4B). The proteins that negatively correlated with the severity of the stroke were functionally associated with hemostasis and focal adhesion processes (Figure 4C).

In line with these results, the levels of 68 proteins were significantly correlated with the ASPECTS score, where high levels of 63 proteins and low levels of 5 proteins were associated with a more severe stroke in terms of the extent of early ischemic changes in the brain (Figure 4D). The functional categorization of this signature showed that the proteins that were positively associated with the severity of the disease (negative correlation with ASPECTS score) were interconnected in a network enriched in biological pathways, such as the innate immune system, neutrophil degranulation, proteasome degradation, and metabolism (Figure 4E). The proteins that were negatively associated with the severity of the stroke (positive correlation with ASPECTS score) were functionally associated with blood clotting cascade and platelet function (Figure 4F).

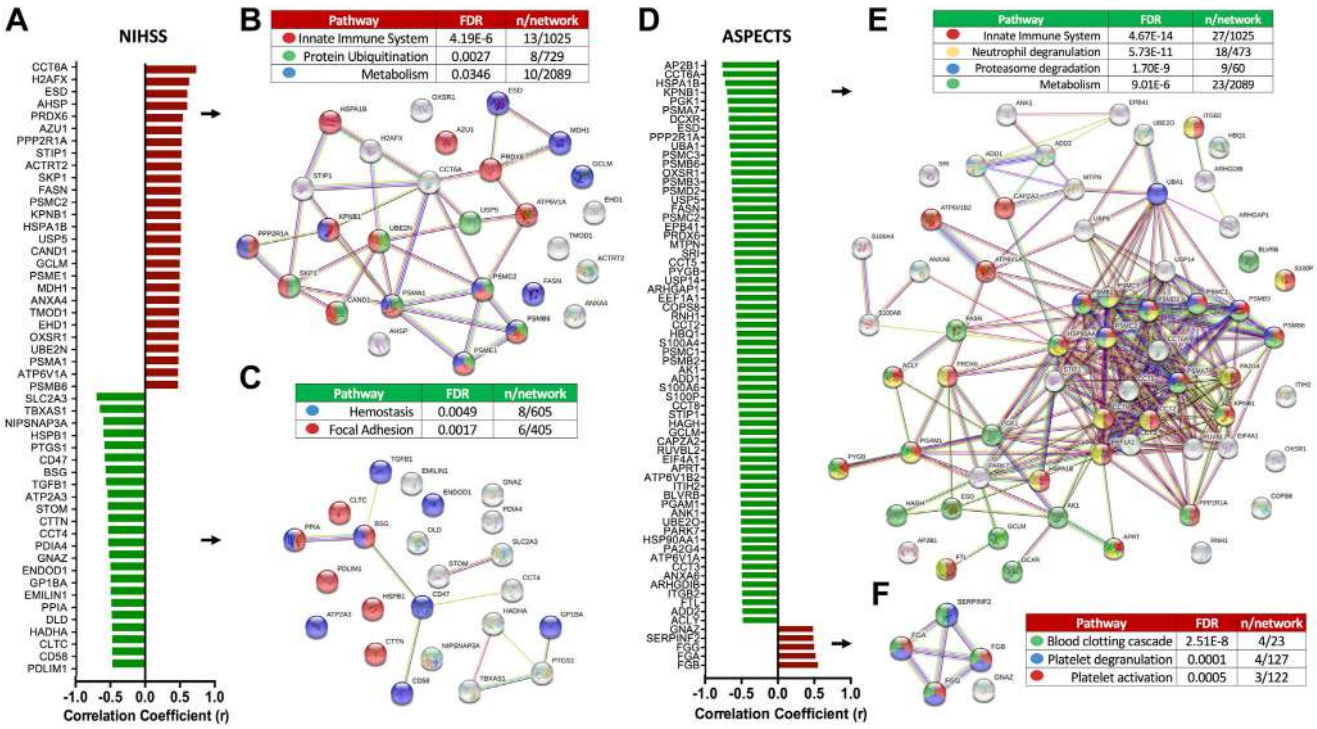


FIGURE 4 Correlation of thrombus protein levels with the severity of the stroke. (A) Pearson correlation analysis of National Institute of Health Stroke Scale (NIHSS) scores and protein levels of the thrombus in patients with stroke. (B, C) Enrichment analysis and network of proteins correlated with NIHSS. (D) Correlation analysis of Alberta Stroke Program Early CT Score (ASPECTS) scores and protein levels of the thrombus in patients with stroke. (E, F) Enrichment analysis and network of proteins correlated with ASPECTS. Red and green colors indicate positive and negative correlations, respectively. FDR, false discovery rate.

Interestingly, 15 proteins were correlated simultaneously with both scores of stroke severity, including GCLM, STIP1, PRDX6, PSMC2, FASN, USP5, ATP6V1A, OXSR1, PSMB6, PPP2R1A, ESD, KPNB1, HSPA1B, CCT6A, and GNAZ, which might be potential targets involved in the onset of more severe strokes.

3.5 | Neutrophil count and activation markers are directly associated with the severity and prognosis of patients who experienced a stroke

Due to the robust presence in the thrombus of proteins related to the innate immune system in general and to neutrophil activity in particular and its association with the severity of the stroke, a new set of analyses was performed to evaluate in detail the role of these cells in the disease in a large independent cohort of patients.

First, we identified an association between the number of neutrophils and the severity as well as with the prognosis of the disease using clinical information of 210 patients who experienced a stroke (Figure 5A, B). Thus, the neutrophil count of patients presenting a severe stroke, characterized by NIHSS of > 15 [14] and ASPECTS of < 7 [15], was significantly elevated compared with those patients with a mild or moderate stroke. Furthermore, the neutrophil count at the time of the stroke was significantly increased in patients exhibiting a worse and more severe prognosis 90 days later (mRS90, >4) [16].

Second, an association among several neutrophil activation markers and the severity and prognosis of the stroke was also recognized using purified neutrophils from 50 patients with stroke of the same independent cohort (Figure 5C–E). Thus, the expression levels of neutrophil activation markers such as CD66B, IL8, PDE4B, and SOD2 were elevated in neutrophils from patients with a high NIHSS and mRS score, most of them showing statistical differences.

In addition, we selected and measured in these cells other neutrophil-related proteins that were both identified in the thrombus and associated with the severity of the stroke, such as CCT2, CCT8, CD47, and KPNB1. We also found a significant relationship with both severity and outcome of stroke after thrombectomy (Supplementary Figure S3).

Finally, we evaluated the levels of circulating elastase (a surrogate marker of NETosis) in the plasma of these patients (Figure 5F, G). Patients with a severe stroke (NIHSS, >15) showed increased levels of circulating neutrophil elastase compared with those with mild or moderate stroke, although this trend was not statistically significant. Patients with a worst prognosis (mRS90 > 4) presented a significant increase in the circulating neutrophil elastase levels at the time of the stroke. Thus, our results pointed at a potential association of NETosis bioproduct levels with the severity and prognosis of the stroke.

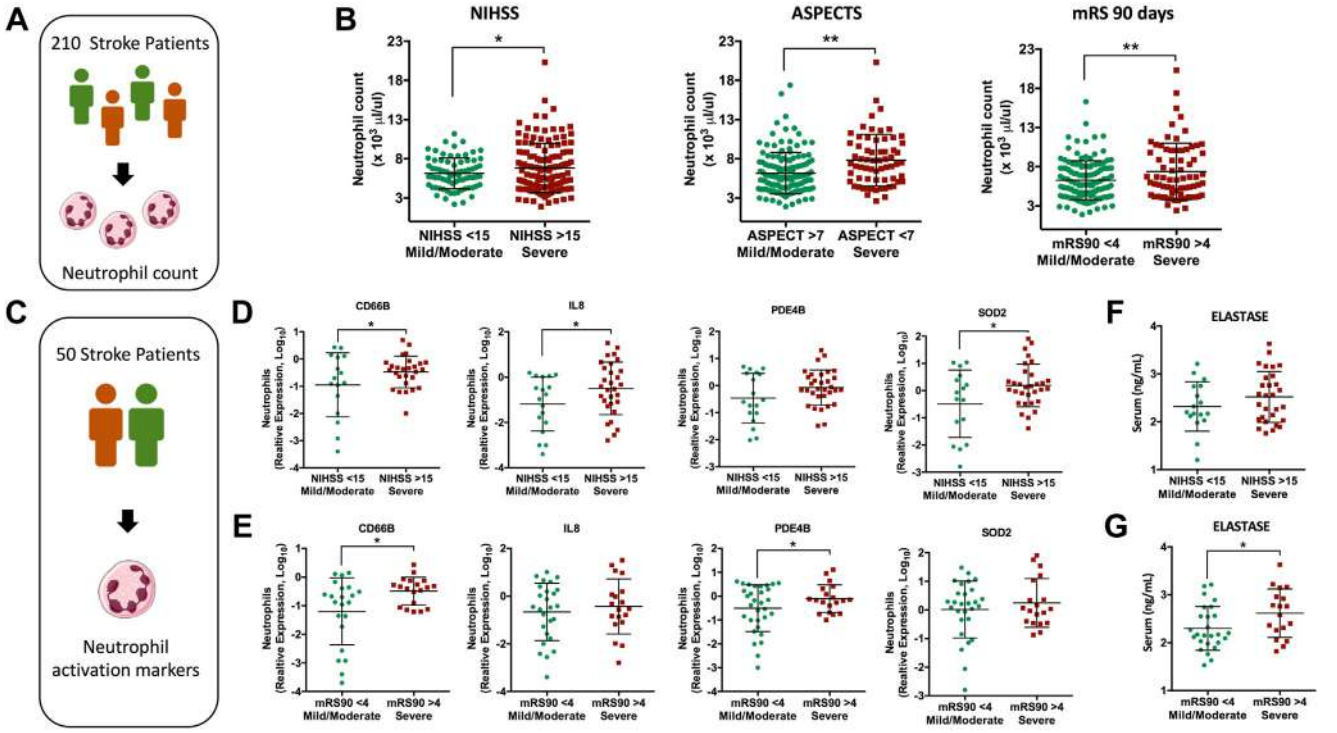


FIGURE 5 Association of neutrophil activation markers with the severity and prognosis of the stroke. (A) Diagram showing the analysis of neutrophil count in 210 patients with stroke. (B) Association of neutrophil counts with National Institute of Health Stroke Scale (NIHSS), Alberta Stroke Program Early CT Score (ASPECTS), and modified Rankin Scale (mRS) scores. (C) Diagram showing the analysis of neutrophil activation markers in 50 patients with stroke. (D, E) Association of neutrophil activation markers in purified neutrophils by RT-PCR and NIHSS scores. (E) Association of neutrophil activation markers in purified neutrophils by RT-PCR and NIHSS and mRS scores. (F, G) Association of circulating levels of neutrophil elastase in the plasma of patients with stroke as a marker of NETosis and NIHSS and mRS scores. mRS90, modified Rankin Scale score 90 days after the event. RT-PCR, real-time polymerase chain reaction. *$p < .05$. ** $p < .01$.

4 | DISCUSSION

In this study, we performed for the first time a proteomic SWATH-MS analysis in thrombi from a cohort of patients who experienced an acute ischemic stroke, which has revealed important insights related to the composition of the clot and biological pathways involved in its etiology, severity, and progression. The analysis has highlighted the relevant influence of the immune system in general and neutrophils in particular on all these processes, which has been confirmed and extended in independent cohorts of patients who experienced a stroke using clinical data and cellular analysis.

The unsupervised analysis of the composition of the thrombus identified >500 hundred proteins, which were categorized into 4 groups according to their biological meaning. The most enriched biological pathways identified in these groups were hemostasis, regulation of actin cytoskeleton, proteasome, and neutrophil degranulation. First, we identified the presence of high levels of well-known proteins involved in the coagulation cascade, such as fibrinogens and coagulation factors, as well as proteins derived from erythrocytes (hemoglobin) and platelets (platelet glycoproteins) [17], which reinforced the role of the SWATH-MS analysis of thrombi as a reliable approach to obtain key information from recognized and novel players participating in the stroke process.

Second, the relevant presence of proteins related to the cytoskeleton, focal adhesion, and tight junctions, such as integrins, myosin, vinculins, filamins, and tubulins, evidenced the role of the cellular component in the thrombus formation, suggesting a key role of these proteins in the structural maintenance of the clot [18].

Another interesting group of proteins enriched in the thrombus are those related to the ubiquitin-proteasome system (UPS). The UPS is a major protein degradation system in eukaryotes that identifies and degrades aberrant proteins (misfolded proteins or normal short-lived proteins) [19]. It has been proposed that the UPS may be part of an inflammatory pathway leading to thrombus formation and, subsequently, stroke [20]. In this sense, our results suggest that the UPS is involved in the molecular mechanisms leading to stroke, since several protein groups identified as most abundant in clots are directly connected to these pathways.

Besides, UPS is not only the main pathway for the degradation of proteins but also the main regulator for maintaining neural development, brain structure, and function [21]. Most stroke thrombi are formed in the periphery and subsequently embolize to the brain. Thus, the proteins identified in the clot reflect the molecular environment of the cells that are part of the thrombus or involved in thrombus development. Hence, the presence of the UPS in the thrombus might also reflect the direct involvement of this system in several pathological

changes triggered by a stroke in surrounding tissues, such as oxidative stress, mitochondrial autophagy, inflammatory response, and hypoxia, which have been directly associated with UPS in other contexts [22]. In line with this, it has also been reported that one of the important features after stroke is the aggregation of ubiquitinated proteins [23]. Thus, the modulation of the UPS has been proposed as a potential therapeutic approach for patients who experienced a stroke [24].

Another key group of proteins integrated in the thrombus is those related to the innate immune system where neutrophils seem to have a leading role, since a lot of proteins related to central neutrophil functions were expressed at high levels in the clot. In that way, extensive literature has found and characterized the presence of neutrophils in thrombi using techniques such as immunohistochemistry [25,26] and electron microscopy [27,28].

Nevertheless, although the role of the immune system in the development of stroke has been previously pointed out [29], the consideration of these cells as targets to be therapeutically modulated in stroke has not been tackled in depth.

Other proteins involved in cellular metabolism and other neurological diseases were also present in the thrombus. The strong correlation among all these proteins also suggested that the development of the clot might be directly linked to a complex and coordinated process in which many different biological mechanisms are intimately interconnected at the same time.

Once we characterized the composition of thrombi, we performed an unsupervised cluster analysis to test the capacity of the proteomic content to stratify patients with stroke. This analysis revealed distinctive and critical clinical profiles associated with the origin (cardioembolic and atherothrombotic), severity (NIHSS and ASPECTs), and prognosis of the disease (mRS90). This is the first evidence showing that the high-throughput analysis at the protein level of thrombi from patients with stroke has the potential to identify patients with important clinical features, which might influence the clinical management of these patients in a more personalized manner.

With the aim of gaining more insight into the etiology and development of stroke and identifying novel players involved in these processes, we further performed a comparative analysis of the protein content of thrombi from patients who had suffered cardioembolic and atherothrombotic strokes, revealing a group of proteins differentially expressed between both groups. Among them, several proteins were upregulated in atherothrombotic thrombi, which has been previously associated with the development of atherosclerosis, including an autophagy-related protein (ATG3) [30,31], an erythrocyte-Rh blood group protein erythrocytes (RHD) [32], a key protein involved in the coagulation cascade like fibrinogen, a glucose transporter (GLUT-1 and SLC2A1) [33], a chloride transporter channel (CLIC-4) [34], and a keratin protein (KRT1) [35]. Furthermore, several proteins with key recognized functions in stroke and cardiovascular disease were upregulated in thrombi from cardioembolic etiology, including 2 SERPIN family members (SERPINC1 and SERPINA3) [36,37], a modulator of plasminogen activity (vitronectin) [38], a serum ferroxidase (ceruloplasmin) [39], 2 apolipoproteins (APOA1 and CLU or apolipoprotein-J) [40,41] and an endothelial nitric oxide synthase binding protein (EEF1A1) [42].

The signature integrated by these specific and differentially expressed proteins showed the capacity to clearly distinguish patients according to the etiology of the stroke, which was also linked to clinical cardiovascular risk factors such as obesity, hypertension, smoking, and diabetes. In this regard, a very recent meta-analysis by Huang et al. [43] aimed to delineate the association of brain clot composition with stroke etiology and found that fibrin composition is significantly higher in strokes of cardioembolic origin. Nevertheless, it is well known that the process of thrombus formation is much more complex, so new studies have further described in detail the role of von Willebrand factor, neutrophil extracellular traps (NETs), and DNA [44].

The different composition of proteins found in the thrombi according to its differential etiology suggests that a stroke might occur because of distinctive pathogenic mechanisms, which might have important implications associated with a more tailored clinical management. Other researchers have tried to identify proteins in the blood as biomarkers for the etiologic diagnosis of stroke with poor sensitivity [45]. Thus, the analysis of markers in a unique type of sample such as thrombi of patients who experienced a stroke seems to represent a better strategy for this purpose, as shown in the present study.

Our results are in line with very recent studies, such as the report by Rossi et al. [20], who used label-free quantitative LC-MS/MS on formalin-fixed paraffin-embedded thrombectomy samples, and identified a similar pattern of proteins.

Similarly, a discovery-based SWATH-MS proteomic approach to identify blood-based protein biomarkers to diagnose stroke [46] determined that the most common significant pathways in stroke cases involved complement and coagulation cascades, platelet degranulation, immune-related processes, acute phase response, lipid-related processes, and pathways related to extracellular space and matrix.

A deeper analysis of the biological pathways and proteins associated with the severity of the stroke was also performed. This analysis revealed that patients who had suffered a more severe stroke presented higher levels of proteins related to neutrophil and innate immune system, cellular metabolism, and proteasome and lower level of proteins associated with the hemostasis and structural scaffold. The identification of an independent association of these processes with 2 different scores of severities that consider computed tomography images (ASPECTS) on the one hand and neurologic function on the other (NIHSS) reinforced and further supported our findings.

Several potential proteins were directly associated at the same time with both scores of stroke severity (NIHSS and ASPECTS), which highlighted their potential role as biomarkers of disease. Among them, we identified relevant proteins that have been previously associated with stroke, including proteins involved in oxidative stress processes (GCLM, glutamate-cysteine ligase regulatory subunit [47]; PRDX6, peroxiredoxin-6 [48]; stress-induced STIP1, stress-induced-phosphoprotein 1 [49]; HSPA1B, heat shock 70 kDa protein 1B [50]; lipid metabolism FASN, fatty acid synthase [51]; and intracellular

signaling OXSR1, serine/threonine-protein kinase OSR1 [52]). Moreover, novel proteins biomarkers that have not been previously related to stroke disease were also correlated with the stroke severity, including proteasome components (PSMB6 and USP5), proteases (PSMC2), ATP enzymes (ATP6V1A), glutathione hydrolases (ESD), importins (KPNB1), chaperones (CCT6A), and transmembrane signaling proteins (GNAZ). Altogether, these proteins might open new opportunities to further evaluate in depth their roles in the pathogenesis of the disease, having the potential to be therapeutically targeted.

Since a large number of proteins derived from neutrophils were highly expressed in the thrombus and strongly associated with the severity of the stroke, we explored in detail their involvement and use as potential biomarkers of disease in an independent and extensive cohort of patients. First, using retrospective clinical data, we identified that the levels of these cells at hospital admission were associated not only with the severity (NIHSS and ASPECTS) but also with the prognosis of the stroke 90 days later (mRS90). This is an interesting result, validated in >200 patients, that might have translational relevance, since the detection of the neutrophil count in the peripheral blood of patients who experienced stroke who reach the emergency hospital unit might represent an easy, quick, and accessible tool that can contribute to improve their clinical management. In line with our data, in humans, an increase in neutrophil counts has been associated with symptomatic carotid artery stenosis, cerebral microembolization, and hypoechoic unstable carotid plaques [53]. In the same way, previous works have shown that in patients with stroke treated with mechanical thrombectomy, neutrophil and lymphocyte counts are dynamic parameters associated with hemorrhagic complications and long-term outcome [54–56].

Finally, we also demonstrated that activation markers of purified neutrophils (*CD66B*, *IL8*, *PDE4B*, and *SOD2*) and circulating biomolecules derived from key neutrophils-pathological processes associated with the development of cardiovascular disease such as NETosis (neutrophil elastase), were correlated with both the severity and progression of the stroke.

Moreover, these associations were further identified in neutrophil-related proteins present in the thrombi, which have been demonstrated to be involved in immune cell infiltration and inflammatory processes mediated by NFKB signaling [57–59].

In the last few years, the role of neutrophils as central players in clot formation has increased. Thus, the depletion of neutrophils in mice reduced thrombus formation [60].

The main pathogenic mechanisms associated with the development of cardiovascular diseases include platelet interactions [61] and NET formation [62], which release DNA, tissue factor, and proteases that activate the coagulation cascade [60,63]. Besides, neutrophils have been proven to contribute to both the formation and rupture of the atherosclerotic plaque [64]. Accordingly, a relevant role of activated neutrophils and NET formation in ischemic stroke has been demonstrated, so that lower values of CD66b in thrombi have been independently associated with greater improvement of NIHSS after mechanical thrombectomy [65]. In addition, previous studies evaluating NETs in the plasma of patients who experienced an acute ischemic stroke identified, at onset, high levels of cell-free DNA or citH3—the most specific marker of NETs—which were independently associated with increased severity and mortality in these patients [66].

Moreover, histologic analysis of thrombi from patients who experienced an ischemic stroke that evaluated their immune cell composition and NETs content showed that NET abundance in thrombi was associated with poor outcome scores based on the NIHSS postassessment score and mRS until 90 days [67].

In this framework, our results are the first that further identify in the thrombi using SWATH technology, a large number of proteins derived from neutrophils, which are highly expressed and strongly associated with the severity of the stroke, thus reinforcing the potential involvement of neutrophils as pivotal cells in its pathogenesis. Thus, neutrophils might represent potential biomarkers for monitoring the development and outcome of the disease.

All in all, our results, in combination with other analytical approaches developed for patients who experienced a stroke, suggest that the clot proteome might reflect systemic aspects of the patients that in turn influence stroke severity and progression, even after the removal of the clot.

Cardioembolic clots often occur due to a new onset of a heart arrhythmia, typically atrial fibrillation, and large artery clots tend to result from the embolization of atherosclerotic plaque from a large artery source, commonly the carotid arteries. The cellular and molecular signatures of the clots, formed as a result of these very different underlying conditions, have been demonstrated to be recognizable and identifiable by distinctive technological approaches. Thus, a higher percentage of white blood cells in the thrombus was associated with cardioembolic etiology. Hence, white blood cell–mediated immunologic and coagulatory processes may play a key role in thrombus formation and pathogenesis of stroke [68].

Accordingly, protein composition has been demonstrated in this study to also be distinctive and most probably derived from the clot composition and abundance of different blood cell types. The identification of a high number of proteins related to neutrophil activity which, in turn, were related to stroke outcome, might be a clear example. Nevertheless, this aspect deserves further investigation.

When engaging a clot with a stent-retriever or suction catheter, optimal outcomes are likely best achieved when the entirety of the thrombus can be retrieved in one pass. Multiple passes indicate that the clot adheres to the vessel or is fragmented, which could put the patient at risk of distal emboli and less-than-optimal recanalization outcomes [69]. It has been shown that the immunohistologic content of the clot influences the mechanical characteristics of thrombi and, thus, affects the ability of a stent-retriever device or suction catheter to engage it [70]. Hence, in the present study, we found that patients with first-pass recanalization were mostly belonging to cluster 3, which showed the best outcome. These patients further displayed a distinctive protein profile in relation to clots from clusters 1 and 2, thus pointing at the distinctive abundance of blood cell types and, consequently, the proteins associated as main influencers, as explained above, which might eventually impact the stroke outcome.

Although new studies are needed to confirm and expand our findings, overall, our data thoroughly characterized the proteomic composition of the thrombus from patients who experienced an acute ischemic stroke, providing new insights into pathways and players involved in the etiology, severity, and prognosis of the stroke. The systematic analysis of thrombi also showed the capacity to stratify patients according to crucial and distinctive clinical profiles. The prominent role of the innate immune system and neutrophils identified might pave the way for the future development of new clinical tools, biomarkers, and therapeutic approaches in patients who experience a stroke.

AUTHOR CONTRIBUTIONS

C.P.-S., A.I.-C., M.L.-T., L.M.-B., and E.C.-G. developed the proteomic and transcriptomic analysis and solved technical problems. F.V., R.O., J.M.-E., and J.O. followed up with patients and contributed useful discussion and suggestions. C.L.-P., C.P.-S., N.B., A.I.-C., M.L.-T., and L.M.-B. performed the statistical analysis and discussed the results. C.L.-P., C.P.-S., R.O., and F.V. directed and coordinated the study, designed the experiments, analyzed the data, and wrote the manuscript.

DECLARATION OF COMPETING INTERESTS

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

The online version contains supplementary material available at https://doi.org/10.1016/j.jtha.2023.04.015