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ORIGINAL ARTICLE

Stroke emboli from patients with atrial fibrillation enriched with neutrophil extracellular traps

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Abstract

Background: Recent literature has demonstrated remarkable heterogeneity in the composition of acute ischemic stroke (AIS) emboli, which may impact susceptibility to therapy.

Objectives: In this study, we explored differences in proteomic composition of retrieved embolic material from patients with stroke with and without atrial fibrillation (AF) (AF+ and AF−, respectively).

Methods: The full proteome of retrieved thromboembolic material from 24 patients with AIS was obtained by mass spectrometry. Known marker proteins were assigned groups representing broad classes of embolus components: red blood cells, platelets, neutrophils, eosinophils, histones, complement, and other clotting-associated proteins (eg, fibrinogen). Relative protein abundances were compared between AF+ and AF− samples. Functional implications of differences were explored with gene set enrichment analysis and Gene Ontology enrichment analysis and visualization tool.

Results: One hundred sixty-six proteins were differentially expressed between AF+ and AF− specimens. Eight out of the 15 neutrophil proteins ($P < .05$; fold change, >2) and 4 of the 14 histone proteins were significantly enriched in AF+ emboli ($P < .05$; fold change, >2). Gene set enrichment analysis revealed a significant representation of proteins from published neutrophil extracellular trap (NET) proteomic gene sets. The most significantly represented functional Gene Ontology pathways in patients with AF involved neutrophil activation and degranulation ($P < 1 \times 10^{-7}$).

Conclusion: The present analysis suggests enrichment of NETs in emboli of patients with stroke and AF. NETs are a significant though understudied structural component of thrombi. This work suggests not only unique stroke biology in AF but also potential therapeutic targets for AIS in this population.

KEYWORDS
atrial fibrillation, neutrophil extracellular trap, proteomics, stroke, thrombosis

Essentials

- Atrial fibrillation is a significant cardiac risk factor for acute ischemic stroke.
- We studied the proteomic composition of stroke clots with and without atrial fibrillation.
- Neutrophil extracellular traps made of DNA were enriched in atrial fibrillation clots.
- This suggests a unique treatment target for stroke in atrial fibrillation patients.

1 | INTRODUCTION

Acute ischemic stroke (AIS) remains a major cause of death and disability despite significant active research. First-line recanalization with the timely use of the recombinant intravenous (i.v.) thrombolytic tissue plasminogen activator (tPA; "alteplase") can convert clot-bound plasminogen into active plasmin and lyse thrombus fibrin [1–6]. However, given its stringent eligibility criteria for risk of hemorrhage [7], only 10% of patients are eligible for tPA [1]; further, only about half of those who receive it demonstrate successful reperfusion [1,8].

Since 2015, multiple studies have shown remarkable benefit from using transarterial mechanical thrombectomy in cases of large vessel occlusion when tPA fails or is not indicated [9–12]. However, only about 10% of patients presenting within the first 24 hours of symptoms were eligible for endovascular therapy [13]. Precluding any questions of eligibility are issues of access, however, as mechanical thrombectomy is not available at all hospitals. Furthermore, even when it is available, and patients are eligible, successful recanalization, as defined by thrombolysis in cerebral infarction scores of 2b or 3, is not achieved in up to 20% of cases [14], highlighting the need for more effective and broadly applicable pharmacologic interventions.

Numerous factors are known to affect thrombus susceptibility to thrombolysis with tPA. In particular, more proximal [15–17], longer [15,16], and less permeable thrombi [1,16] have been found to be especially resistant to i.v. thrombolysis. Thrombus composition may be another factor that influences susceptibility to our current "one size fits all" pharmacologic and interventional therapy.

Endovascular thrombectomy has enabled direct study of retrieved thrombemboli, but the literature to date is largely comprised of histopathologic studies. Although they have yielded fundamental insights into thrombus microstructure and macrostructure [18–21], they have not changed treatment paradigms. In contrast, only 3 studies to date have leveraged mass spectrometry in the study of AIS thrombus proteomes and collectively validated the use of this technique in exploring thrombus composition [22–24].

Atrial fibrillation (AF) is not only a significant risk factor for AIS [25] but also an independent predictor for worse outcomes with tPA thrombolysis [26–28]. While numerous studies have demonstrated differences in thrombus composition as a function of etiology (ie, cardioembolic, large-artery atherosclerotic, etc.), our understanding of specific differences in thrombus pathophysiology and their significance in treatment success remains incomplete. In this study, we examine the proteomic composition of retrieved AIS thrombi as it relates to AF to develop a clearer mechanistic understanding of AIS pathophysiology with this etiology.

2 | METHODS

2.1 | Sample and clinical data collection

This study was conducted with approval from our institutional review board and patient consent.

Whole thromboemboli from 24 patients with AIS were collected via mechanical thrombectomy by aspiration or stent retriever at the University of Rochester Medical Center between May and October 2021. All samples were kept moist and promptly transferred to storage in a cryotube in a −80 °C freezer until utilization for mass spectrometry. A full sample collection and processing schema is shown in Figure 1.

Attempted thrombolysis prior to definitive thrombus extraction by i.v. or intra-arterial (IA) recombinant tPA was noted but was not treated as an exclusion criterion. Finally, a retrospective chart review was conducted to identify those patients comorbid with either chronic or new-onset AF at the time of their stroke, along with data pertinent to their medical histories, medication, and clinical outcomes. For the purpose of this study, we did not differentiate between paroxysmal and continuous AF but rather differentiated between those patients in active AF at the time of presentation and those who did not have any arrhythmia. A panel of the first laboratory values after admission to the hospital were also recorded.

2.2 | Sample preparation and mass spectrometry

Sample preparation and processing were done per our institutional Mass Spectrometry Resource Laboratory using the following protocol. Samples were rinsed twice in 1× phosphate buffered saline (PBS) and then solubilized by adding 1 mL of 5% sodium dodecyl sulfate, 100 mM triethylammonium bicarbonate buffer (TEAB), and sonicated (Qsonica) for 5 cycles, with 1-minute intervening resting periods on ice. Lysates were centrifuged at 15,000 × *g* for 5 minutes to precipitate debris before the supernatant was collected. Protein concentration was determined by bicinchoninic acid assay (Thermo Fisher Scientific), after which samples were diluted to 1 mg/mL in 5%

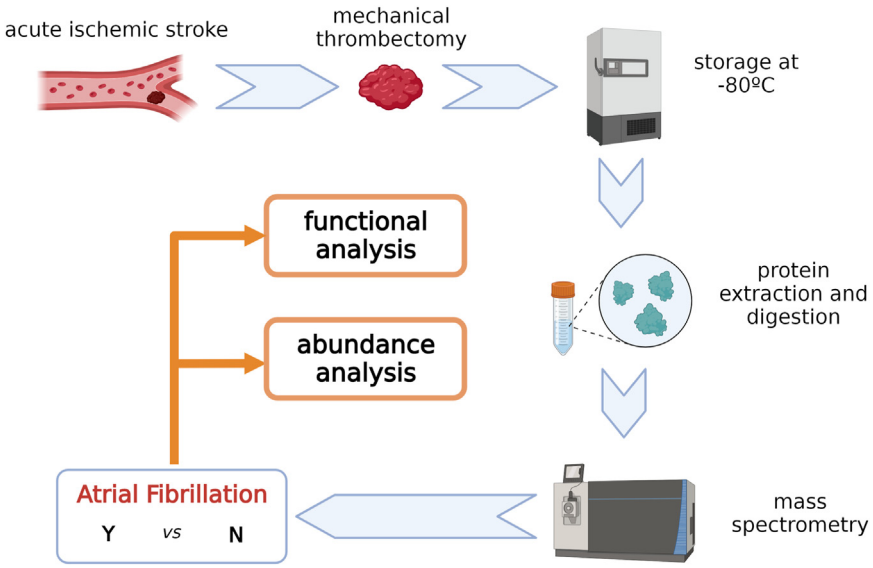

FIGURE 1 Acute ischemic stroke thrombus retrieval and processing schema. N, no; Y, yes.

sodium dodecyl sulfate and 50 mM TEAB. Twenty-five micrograms of protein from each sample was reduced with dithiothreitol to 2 mM and incubated at 55 °C for 60 minutes. Iodoacetamide was added to 10 mM and incubated in the dark at room temperature for 30 minutes for protein alkylation. Phosphoric acid was added to 1.2%, followed by 90% methanol and 100 mM TEAB. The resulting solution was added to S-Trap micros (ProtiFi) and centrifuged at 4000 × *g* for 1 minute. The S-Traps containing trapped protein were washed twice by centrifuging through 90% methanol, 100 mM TEAB. One microgram of trypsin was prepared in 20 µL of 100 mM TEAB and added to the S-Trap, followed by an additional 20 µL of TEAB to ensure the sample did not dry out. The cap to the S-Trap was screwed loosely to ensure the solution was not pushed out of the S-Trap during digestion. Samples were placed in a humidity chamber at 37 °C overnight before the S-Trap was centrifuged at 4000 × *g* for 1 minute to collect the digested peptides. Sequential additions of 0.1% trifluoroacetic acid in acetonitrile and 0.1% trifluoroacetic acid in 50% acetonitrile were added to the S-Trap, centrifuged, and pooled. Samples were frozen and dried in a SpeedVac (Labconco) and then resuspended in 0.1% trifluoroacetic acid prior to analysis. Peptides from each fraction were then processed with a Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific).

2.3 | Mass spectrometry data processing

The raw data were processed with DIA-NN version 1.8.1 (https://github.com/vdemichev/DIA-NN) to identify proteins and perform label-free quantitation [29]. Of the 1797 total proteins identified, 93 known marker proteins were sorted into groups representing broad classes of embolus components: red blood cells (RBCs), platelets, polymorphonuclear neutrophils, eosinophils, histones, complement proteins, and other clotting-associated proteins (eg, fibrinogen). All marker proteins and categories are displayed in the Supplementary Table S1.

2.4 | Abundance analysis

As the final samples processed for mass spectrometry were normalized to a final concentration of 1 mg/mL, the relative abundance of individual proteins, signifying the proportional representation of a given protein within the overall digested sample, was compared between the thrombi of patients with and without AF. This was done using Wilcoxon rank sum tests (RStudio 4.2.0, RStudio Team) comparing relative protein abundances between thrombi from patients with and without AF with a cutoff for significant differences in enrichment set at an alpha of 0.05. The comparison for each protein was represented by a descriptive statistic, the $\log_2$-fold change (FC) in relative abundance for each protein, calculated by (median relative abundance in AF+ thrombi)/(median relative abundance in AF− thrombi). Thus, a $\log_2$-FC of 1 for a given protein represents a 2× greater median relative abundance in thrombi from patients with AF, and a $\log_2$-FC of −1 represents 0.5× greater median relative abundance in thrombi from patients with AF. Thus, all proteins with both a $\log_2$-FC either >1 or less than −1 and a *P* value <.05 were considered to be either significantly enriched or depleted in thrombi from patients with AF.

2.5 | Functional analysis

Functional implications of identified differences in relative protein abundance were explored in 2 ways. First, gene set enrichment analysis (GSEA) was utilized for pathway and ontology analyses [30,31] with a false discovery rate (FDR) of <0.10 set as the cutoff for significance. Published proteomes in the current literature for RBCs, platelets, and neutrophil extracellular traps (NETs) generated *ex vivo* were used as input gene sets for comparison [32–36]. Of the 3 NET protein sets utilized, 2 were generated from *ex vivo* neutrophils sourced from healthy patient controls [35,36], while only the third

involved data from healthy patient controls in addition to those with systemic lupus erythematosus and lupus nephritis [34]. Thus, the overall NET profile that our analysis is capable of identifying is most reflective of that of neutrophils sourced from healthy patients.

Second, the Gene Ontology (GO) enrichment analysis and visualization tool was used to identify highly represented GO terms in thrombi retrieved from patients with AF [37]. Two unranked lists were provided for analysis, with a "target set" of the proteins found to be significantly enriched or depleted ($\log_2$-FC > 1 or < −1 with $P < .05$) in patients with AF and a "background set" of all of the 1797 proteins identified through mass spectrometry across our samples. An uncorrected alpha of 1×10^{-3} was chosen as the threshold for significance for results of the analysis before correction for multiple hypothesis testing, with a maximal FDR of 0.01, corresponding to a $-\log_{10}$(q value) of >2 [38]. The majority of our discussion, however, focuses on results with an FDR of <0.001, or $-\log_{10}$(q value) >3.

3 | RESULTS

3.1 | Patient demographics

The 24 patients with AIS included in the study represented all of the patients between May and October of 2021 from whom thrombus was obtained during thrombectomy. The average ± SD age of all patients was 74 ± 13 years, with 67% being male. All patients identified as White. The majority (75%) of patients received tPA in the course of their treatment (i.v., IA, or both), and all patients were recanalized to a final thrombolysis in cerebral infarction score of 2b/3 after thrombectomy. Thirteen of the total 24 patients in this study had AF (AF+) discovered on electrocardiogram during their admission, while the remaining 11 did not (AF−), with the majority of these classified as large vessel atheroembolic strokes. Twelve out of the 13 AF+ strokes were determined to be cardioembolic related to AF, with the remaining stroke classified as suspected atheroembolism. Of note, all 24 patients included in this study were COVID-negative at the time of their embolectomy. Additionally, there were no significant differences in the proportion of patients that received tPA between those with or without AF. Overall, there were no significant differences in cardiovascular or other risk factors or blood cell values (eg, neutrophil-to-lymphocyte ratio) between the 2 populations (Table 1).

3.2 | Protein abundance analysis demonstrates differential enrichment of numerous proteins, including histone and neutrophil proteins, in AF stroke thrombi

One thousand seven hundred ninety-seven protein signatures were identified from the total 24 retrieved specimens. Of these, median relative abundance values significantly differed between AF+ and AF− groups for a total of 166 proteins. Proteins most enriched in specimens from the AF+ group included tyrosine-protein kinase FGR (Z = −2.55; $P = .005$), peptidoglycan recognition protein 1 (Z = −1.69; $P < .05$), HLA class I histocompatibility antigen, B alpha chain (Z = −2.05; $P = .02$), galectin-10 (Z = −1.97; $P = .02$), and nonsecretory ribonuclease (Z = −2.02; $P = .02$), among many others. The 2 proteins enriched in specimens from the AF− group included the interferon-induced GTP-binding protein Mx1 (Z = −1.66; $P < .05$) and tripartite motif-containing protein 72 (mitsugumin-53) (Z = −1.75; $P = .04$). The most differentially enriched proteins are listed in Table 2, and a complete list of all differentially enriched proteins may be found in Supplementary Table S2.

Volcano plots showed no trends in the clustering of RBC proteins, complement proteins, or clotting-associated proteins (Figures 2A–C). Platelet factor 4 variant 1 was the only platelet protein significantly enriched in the AF+ group (Z = −1.73; $P = .04$) (Figure 2D). Multiple neutrophil proteins, including S100A8, S100A9, azurocidin, myeloperoxidase, and proteinase 3, were significantly enriched in the AF+ group (Z < −1.65 for all; $P < .05$ for all) (Figure 2E). Several histone proteins, (H1-10, H2AC4, H2BC11, and H3C1) were also significantly enriched in the AF+ group (Z < −2.02; $P < .05$ for all) (Figure 2F). Finally, all noted eosinophil proteins (galectin-10, eosinophil-derived neurotoxin, eosinophil peroxidase, eosinophil cationic protein, and integrin subunit alpha M) were also significantly enriched in the AF+ group (Z < −1.81 for all; $P < .05$ for all) (Figure 2G).

3.3 | Functional analysis demonstrates enrichment of NET pathways in AF stroke thrombi

Evaluation of the biological processes represented in the embolic material from AF+ and AF− samples was done using GSEA. Evaluation of hallmark Molecular Signatures Database gene sets revealed significant enrichment of activated leukocyte components in AF+ samples (Hallmark_IL2_STAT signaling, FDR = 0.035; Hallmark_Allograft_Rejection, FDR = 0.143), with 9 gene sets characteristic of myeloid cell types all with FDRs below 0.05 [39]. AF+ emboli were additionally found to be strongly enriched for the NET proteins identified in the 3 *ex vivo* studies available at the time of analysis [34–36]. All 3 NET protein sets resulted in FDRs < 0.25 upon multiple GSEA runs, with 2 of the sets exhibiting FDRs within the range of 0.03 to 0.07, indicating a high degree of concordance between the AF+ group and published NET proteomic gene sets (Figure 3A–C). No statistically significant differences were identified in platelet, fibrin, and RBC content and processes between AF+ and AF− samples using gene sets from either transcriptomic or proteomic studies [32,33,39].

This analysis was validated by independent functional analysis of the proteins significantly represented in the AF+ group using the GO enrichment analysis and visualization tool. When analyzed against the proteomic background of all 1797 signatures identified, 77 Biological

TABLE 1 Patient characteristics for both the atrial fibrillation positive and negative groups.

	Total				AF+				AF−				Fisher's exact test	Mann–Whitney U-test	
	N = 24				*n* = 13		54.2%		*n* = 11		45.8%				
	n	% (total)	Mean	SD	n	% (AF+)	Mean	SD	n	% (AF−)	Mean	SD	*P* value	Z score	*P* value
Demographics															
Male sex	16	67	-	-	8	62	-	-	8	73	-	-	.68	-	-
Race/ethnicity: White	24	100	-	-	13	100	-	-	11	100	-	-	-	-	-
Age	-	-	74	13	-	-	75	13	-	-	73	14	-	0.17	.87
Medical history															
Smoking	14	58	-	-	7	54	-	-	7	64	-	-	.70	-	-
Pack years	-	-	16	18	-	-	15	20	-	-	16	16	-	0.37	.71
Hypertension	20	83	-	-	11	85	-	-	9	82	-	-	1.00	-	-
Diabetes	10	42	-	-	5	38	-	-	5	45	-	-	1.00	-	-
Carotid stenosis	5	21	-	-	2	15	-	-	3	27	-	-	.63	-	-
Coronary artery disease	11	46	-	-	7	54	-	-	4	36	-	-	.44	-	-
Overweight (25 < BMI < 30)	10	42	-	-	7	54	-	-	3	27	-	-	.24	-	-
Obese (BMI ≤ 30)	6	25	-	-	3	23	-	-	3	27	-	-	1.00	-	-
Prior DVT/PE	3	13	-	-	1	8	-	-	2	18	-	-	.58	-	-
Myocardial infarction	4	17	-	-	3	23	-	-	1	9	-	-	.60	-	-
Patent foramen ovale	1	4	-	-	0	0	-	-	1	9	-	-	.46	-	-
HFrEF (LVEF < 45%)	1	4	-	-	0	0	-	-	1	9	-	-	.46	-	-
Prior stroke	5	21	-	-	2	15	-	-	3	27	-	-	.63	-	-
Inflammatory/autoimmune disease	5	21	-	-	4	31	-	-	1	9	-	-	.33	-	-
Kidney disease	6	25	-	-	3	23	-	-	3	27	-	-	1.00	-	-
Prior malignancy	11	46	-	-	5	38	-	-	6	55	-	-	.68	-	-
Active malignancy	3	13	-	-	1	8	-	-	2	18	-	-	.58	-	-
Medications															
Aspirin	14	58	-	-	9	69	-	-	5	45	-	-	.41	-	-
ADP inhibitor	3	13	-	-	2	15	-	-	1	9	-	-	1.00	-	-
Heparin	2	8	-	-	0	0	-	-	2	18	-	-	.20	-	-

(Continues)

TABLE 1 (Continued)

	Total				AF+				AF−				Fisher's exact test	Mann–Whitney U-test	
	N = 24				*n* = 13	54.2%			*n* = 11	45.8%					
	n	% (total)	Mean	SD	n	% (AF+)	Mean	SD	n	% (AF−)	Mean	SD	*P* value	Z score	*P* value
Warfarin	3	13	-	-	3	23	-	-	0	0	-	-	.22	-	-
Direct thrombin inhibitor	0	0	-	-	0	0	-	-	0	0	-	-	NA	NA	NA
Factor Xa inhibitor	2	8	-	-	2	15	-	-	0	0	-	-	.48	-	-
Statin	14	58	-	-	8	62	-	-	6	55	-	-	1.00	-	-
tPA															
i.v. tPA	9	38	-	-	4	31	-	-	5	45	-	-	.66	-	-
IA tPA	10	42	-	-	5	38	-	-	5	45	-	-	1.00	-	-
Any	18	75	-	-	9	69	-	-	9	82	-	-	.65	-	-
Thrombus location															
M1	10	42	-	-	6	46	-	-	4	36	-	-	.70	-	-
M2	5	21	-	-	2	15	-	-	3	27	-	-	.63	-	-
ICA and M1	4	17	-	-	3	23	-	-	1	9	-	-	.60	-	-
M1 and M2	4	17	-	-	2	15	-	-	2	18	-	-	1.00	-	-
Basilar	1	4	-	-	0	0	-	-	1	9	-	-	.46	-	-
Imaging outcome															
HCAS	14	58	-	-	7	54	-	-	7	64	-	-	.70	-	-
Hemorrhage	2	8	-	-	1	8	-	-	1	9	-	-	1.00	-	-
Death	4	17	-	-	1	8	-	-	3	27	-	-	.30	-	-
First pass effect mTICI 2b/3	15	63	-	-	7	54	-	-	8	73	-	-	.42	-	-
Final mTICI 2b/3	24	100	-	-	13	100	-	-	11	100	-	-	NA	NA	NA
LKW to recanalization (min)	-	-	476	316	-	-	518	309	-	-	426	330	-	0.78	.44
ICU LOS (d)	-	-	2	3	-	-	2	2	-	-	3	3	-	0.58	.56
Ventilator LOS (d)	-	-	1	2	-	-	0	0	-	-	2	2	-	1.91	.06
Hospital LOS (d)	-	-	11	14	-	-	9	6	-	-	13	21	-	0.52	.60
Discharge mRS	-	-	3	2	-	-	3	2	-	-	4	2	-	1.30	.19
90-day mRS	-	-	3	2	-	-	3	2	-	-	3	2	-	0.87	.38

(Continues)

TABLE 1 (Continued)

	Total				AF+				AF−				Fisher's exact test	Mann–Whitney U-test	
	N = 24				*n* = 13		54.2%		*n* = 11		45.8%				
	n	% (total)	Mean	SD	n	% (AF+)	Mean	SD	n	% (AF−)	Mean	SD	*P* value	Z score	*P* value
Laboratory tests															
WBC	-	-	9.6	3.1	-	-	10.0	3.0	-	-	9.0	3.0	-	0.61	.54
PMN	-	-	7.3	3.2	-	-	7.6	3.3	-	-	7.0	3.2	-	0.06	.95
LYMPH	-	-	1.4	1.1	-	-	1.4	1.1	-	-	1.5	1.1	-	0.46	.65
PMN/LYMPH	-	-	10.2	14.2	-	-	9.9	13.4	-	-	10.7	15.7	-	1.13	.26
RBC	-	-	4.1	0.8	-	-	4.0	0.6	-	-	4.2	0.9	-	0.93	.35
Hb	-	-	12.7	2.2	-	-	12.7	1.7	-	-	12.8	2.7	-	0.23	.82
HCT	-	-	39.0	6.2	-	-	38.6	5.1	-	-	39.4	7.5	-	0.43	.67
PLT	-	-	207.3	99.4	-	-	232.2	110.2	-	-	177.8	79.9	-	0.93	.35
Calcium	-	-	9.1	0.7	-	-	9.1	0.6	-	-	9.1	0.8	-	0.29	.77
Magnesium	-	-	1.9	0.3	-	-	1.9	0.3	-	-	1.9	0.3	-	0.00	1.00
Creatinine	-	-	1.06	0.45	-	-	0.90	0.28	-	-	1.26	0.54	-	1.97	.05
PT	-	-	13.7	3.7	-	-	15.0	4.6	-	-	12.5	2.3	-	0.79	.43
INR	-	-	1.2	0.3	-	-	1.3	0.4	-	-	1.1	0.2	-	0.71	.48
PTT	-	-	27.9	5.0	-	-	29.9	5.5	-	-	26.3	4.2	-	0.71	.48
Total cholesterol	-	-	144	38	-	-	135	32	-	-	153	43	-	1.05	.29
HDL	-	-	42	12	-	-	44	12	-	-	39	13	-	1.29	.20
LDL	-	-	75	29	-	-	69	25	-	-	82	33	-	0.98	.33
Triglycerides	-	-	133	58	-	-	109	34	-	-	160	69	-	2.06	.04
HbA1c	-	-	6.5	1.6	-	-	5.9	0.7	-	-	7.1	2.1	-	1.60	.11

AF, atrial fibrillation; BMI, body mass index; DVT, deep vein thrombosis; Hb, hemoglobin; HCT, hematocrit; HDL, high-density lipoprotein; HFrEF, heart failure with reduced ejection fraction; IA, intra-arterial; ICA, internal carotid artery; ICU, intensive care unit; INR, international normalized ratio; i.v., intravenous; LDL, low-density lipoprotein; LOS, length of stay; LVEF, left ventricular ejection fraction; mRS, modified Rankin scale; PE, pulmonary embolism; PLT, platelet; PMN, polymorphonuclear neutrophil; PT, prothrombin time; PTT, partial thromboplastin time; RBC, red blood cell; mTICI, modified Thrombolysis in Cerebral Infarction; tPA, tissue plasminogen activator; WBC, white blood cell.

TABLE 2 The 20 most significantly enriched and 2 significantly depleted proteins in the thrombi of patients with atrial fibrillation relative to thrombi of patients without atrial fibrillation.

Enriched in	Protein name	Gene	UniProt ID	Fold change	Z	*P* value
AF+	Tyrosine-protein kinase Fgr	*FGR*	P09769	89.9	−2.55	.005
	Peptidoglycan recognition protein 1	*PGLYRP1*	O75594	26.2	−1.69	.045
	HLA class I histocompatibility antigen, B alpha chain	*HLA-B*	P01889	9.5	−2.05	.020
	Galectin-10 (Gal-10) (Charcot-Leyden crystal protein)	*CLC*	Q05315	8.3	−1.97	.024
	Nonsecretory ribonuclease (eosinophil-derived neurotoxin)	*RNASE2*	P10153	8.1	−2.02	.022
	Carcinoembryonic antigen-related cell adhesion molecule 6 (CD antigen CD66c)	*CEACAM6*	P40199	7.5	−2.45	.007
	Ferritin heavy chain (Ferritin H subunit)	*FTH1*	P02794	7.2	−2.73	.003
	Eosinophil peroxidase	*EPX*	P11678	7.2	−2.23	.013
	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2 (CD antigen CD157)	*BST1*	Q10588	7.0	−2.32	.010
	Nicotinamide phosphoribosyltransferase (NAmPRTase) (Visfatin)	*NAMPT*	P43490	6.4	−2.40	.008
	Long-chain fatty acid transport protein 4	*SLC27A4*	Q6P1M0	6.3	−3.04	.001
	CD82 antigen	*CD82*	P27701	6.1	−1.95	.026
	Charged multivesicular body protein 2a (Chromatin-modifying protein 2a)	*CHMP2A*	O43633	5.8	−2.46	.007
	Protein-arginine deiminase type-4	*PADI4*	Q9UM07	5.4	−2.25	.012
	GAS2-like protein 3	*GAS2L3*	Q86XJ1	5.3	−2.10	.018
	Carcinoembryonic antigen-related cell adhesion molecule 8 (CD67 antigen) (CD antigen CD66b)	*CEACAM8*	P31997	5.3	−2.22	.013
	Nicastrin	*NCSTN*	Q92542	5.2	−3.20	.001
	NPC intracellular cholesterol transporter 2	*NPC2*	P61916	5.0	−2.54	.006
	Ferritin light chain (Ferritin L subunit)	*FTL*	P02792	4.6	−2.52	.006
	Heterogeneous nuclear ribonucleoprotein M	*HNRNPM*	P52272	4.6	−1.93	.026
AF−	Tripartite motif-containing protein 72 (mitsugumin-53)	*TRIM72*	Q6ZMU5	0.4	−1.75	.040
	Interferon-induced GTP-binding protein Mx1	*MX1*	P20591	0.1	−1.66	.048

AF, atrial fibrillation.

Process GO terms were represented in AF+ samples (Figure 4A). Of these, 16 terms met criteria for significance with corrected q-values ($-\log_{10}$[q value] > 2), with 14 of these terms even having $-\log_{10}$(q value) > 3, which corresponds to an FDR of 0.001 (Figure 4B). The most significantly represented Biological GO superfamily was "cell activation," with highly represented subfamilies including "myeloid leukocyte migration," "neutrophil degranulation," "granulocyte activation," "neutrophil activation," and "neutrophil activation involved in immune response" (Figure 4C).

4 | DISCUSSION

Using several reinforcing analytic approaches, this study, for the first time, demonstrates that AF is associated with differences in the proteomic composition of retrieved embolic material from patients with AIS. Grouping proteins based on typical thrombus constituents revealed selective relative enrichment of neutrophil and histone proteins in thrombi from AF+ patients. Gene set analysis of thrombus proteins in AF+ and AF− patients showed a significant representation of NET-specific protein gene sets in the AF+ group based on published NET proteomes. Finally, GO analysis confirmed an overrepresentation of biological processes involved in neutrophil activation and degranulation. Collectively, these data suggest the relative enrichment of NETs in AF+ thrombi as compared to AF− thrombi arising predominantly from large vessel atheroembolisms. This potentially represents a new therapeutic target in a large, discrete subpopulation of patients with AIS.

NETs play an important role in physiologic host defense but are also known to be key facilitators of thrombosis [40–44], with a growing body of literature describing their complex role in facilitating thrombosis in AIS. It thus comes as no surprise that elevated plasma levels of NETs have been detected in the plasma of patients with stroke [45–47]. These

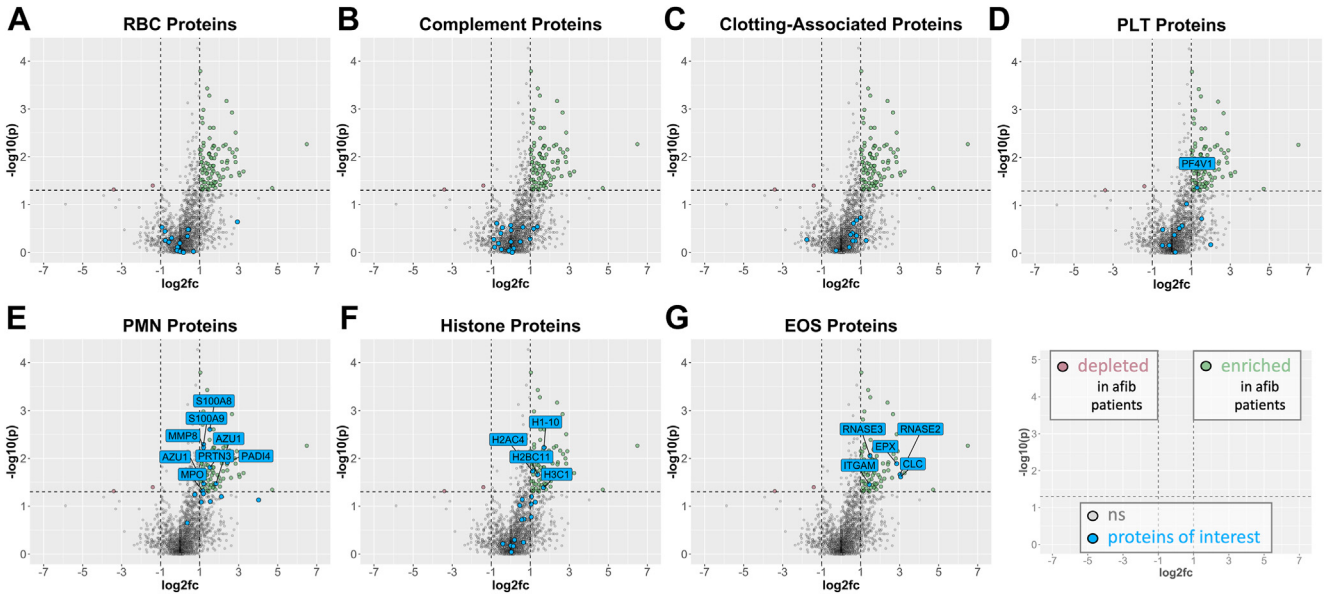

FIGURE 2 Volcano plots demonstrating differential enrichment (green dots) or depletion (red dots) of all 1797 proteins identified by mass spectrometry in patients with atrial fibrillation (afib) using the Wilcoxon rank sum test. The x-axis shows the $\log_2$-fold change (fc), while the y-axis shows $-\log_{10}(P$ value). The vertical dashed lines indicate an fc of ×2 or ×1/2, while the horizontal dashed line indicates a *P* value cutoff of .05. All proteins represented by blue dots indicate those proteins recolored to signify proteins of interest within the category (A) red blood cell (RBC) proteins, (B) complement proteins, (C) clotting-associated proteins, (D) platelet (PLT) proteins, (E) neutrophil (polymorphonuclear neutrophil [PMN]) proteins, (F) histone proteins, and (G) eosinophil (EOS) proteins. ns, not significant.

plasma-derived NETs from patients with stroke display greater thrombogenicity and are less amenable to *in vitro* breakdown by DNase [45]. Complementary to these findings from a study of plasma, histopathologic studies in recent years demonstrate broad enrichment of specific markers of NETs, such as citrullinated H3 in retrieved cerebral thromboemboli [48–50]. Finally, data from *ex vivo* thrombolysis of retrieved thrombi suggests that these detected NETs in cerebral thromboemboli are unlikely to be silent passengers but may instead contribute to resistance to thrombus dissolution by tPA alone, as better thrombus lysis is observed with the cotreatment of specimens with both

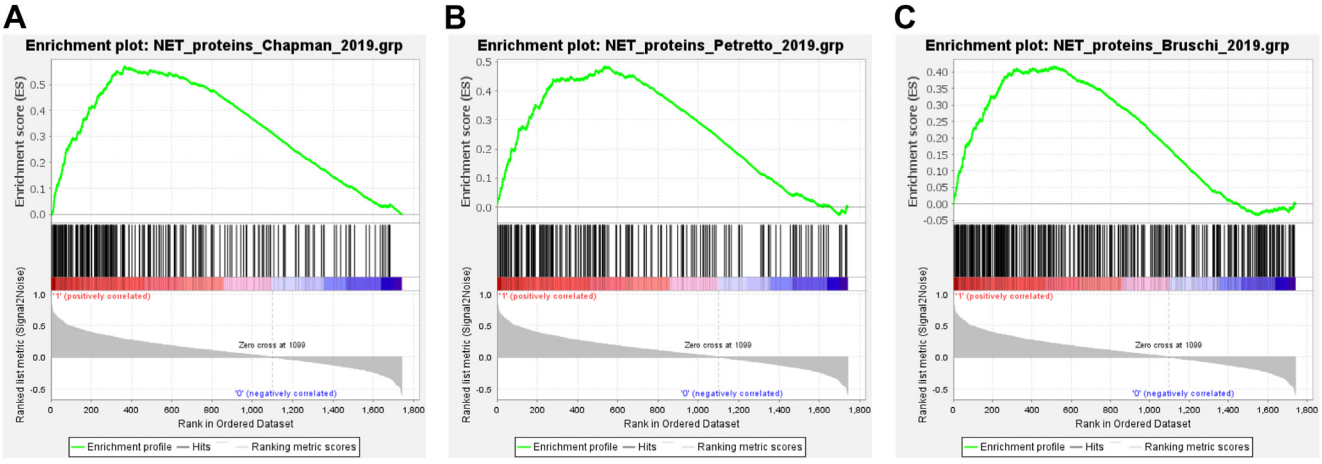

FIGURE 3 Gene Set Enrichment Analysis identifies that atrial fibrillation (AF)+ thrombi in our dataset enrich for neutrophil extracellular trap (NET) proteins derived from *ex vivo* NET studies. Proteins upregulated (red) or downregulated (blue) in AF+ thrombi are plotted along the diagram, with enrichment of a published protein set within the AF+ proteome shown by a greater proportion of black lines (proteins of interest) found towards the left of the diagram. This enrichment is quantified by the enrichment score (ES) plotted in green and validated through multiple hypotheses testing, which generates false discovery rate (FDR) and normalized enrichment score (NES) values. In tests for gene set enrichment in AF+ thrombi, all 3 *ex vivo* NET protein sets met the recommended FDR threshold for significance (FDR, <0.25) and yielded substantial NES above 1.5. (A) Chapman et al. [36] (an FDR of 0.035 and an NES of 1.94), (B) Petretto et al. [35] (an FDR of 0.035 and an NES of 1.88), and (C) Bruschi et al. [34] (an FDR of 0.172 and an NES of 1.61).

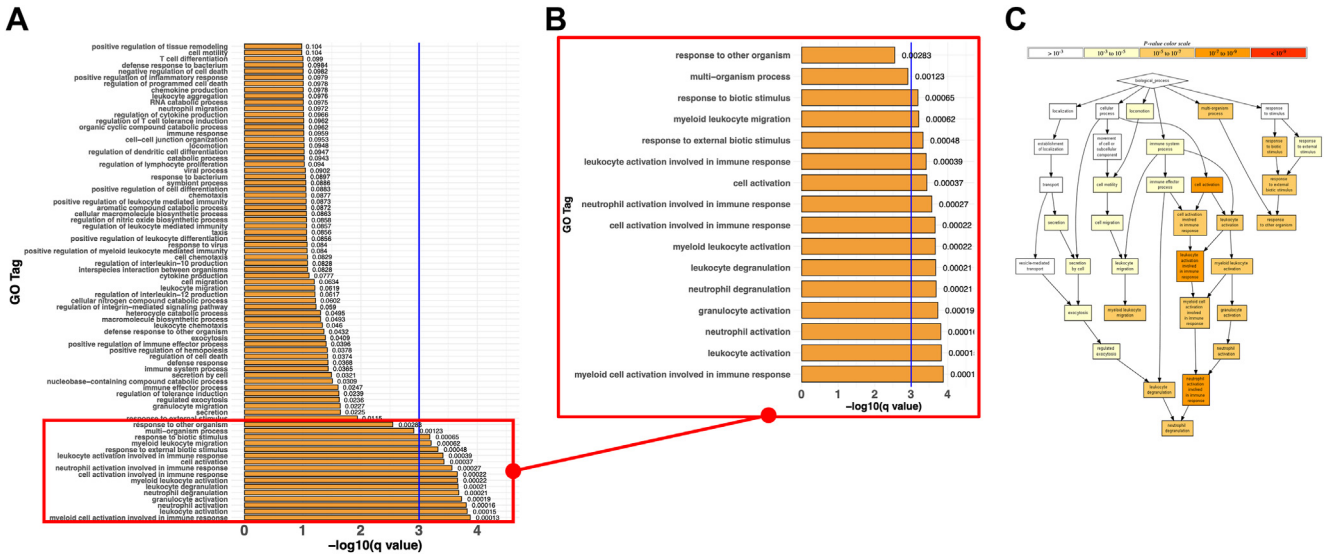

FIGURE 4 Gene Ontology (GO) enrichment analysis and visualization tool analysis of biological processes using all proteins significantly enriched in atrial fibrillation (AF)+ thrombi against the proteomic background of the full dataset of 1797 proteins identified by mass spectrometry. (A) Seventy-seven Biological Process GO terms were overrepresented in AF+ samples (uncorrected $P < 1 \times 10^{-5}$; q value represents corrected *P* value). The vertical blue line represents a $-\log_{10}$(q value) = 3 or false discovery rate = 0.001. (B) Sixteen out of 77 terms met the criteria for significance with Benjamini–Hochberg corrected $-\log_{10}$(q value) > 2, corresponding to a false discovery rate of <0.01, with 14 out of these 16 having a $-\log_{10}$(q value) > 3 (represented by the vertical blue line). (C) The most significantly represented Biological GO superfamily was "cell activation," with highly represented subfamilies suggesting neutrophil activation and degranulation. All 16 boxes with uncorrected $P < 1 \times 10^{-5}$ per the color scale had a corrected $-\log_{10}$(q value) of >3.

DNase 1 and tPA [50,51]. Overall, the recently uncovered role of NETs in AIS through "immunothrombosis" is an area of active research, with the majority of studies on the topic having been published in the last 5 years.

The association between neutrophils, NETs, and AF has previously been reported in the literature. High circulating neutrophil-to-lymphocyte ratios have long been thought to be associated with increased risk of AF [52], as well as greater morbidity and mortality in non-AF stroke [53]. We did not find a statistically significant association between neutrophil-to-lymphocyte ratios and AF in the present dataset, but the large SDs of these laboratory metrics suggest that we may have been limited by sample size. A large body of literature demonstrates the enrichment of NET serum markers in patients with cardioembolic strokes relative to patients with strokes of other etiology [48–50]. Our own finding of NETs in the proteomic analysis of AIS thrombi from patients with AF is mirrored by a recent study of left atrial tissue from patients with AF that shows enrichment of concordant pathways involving neutrophil degranulation as well as the enrichment of NET gene sets [54]. Collectively, the literature suggests a yet unclear relation between neutrophilic inflammation, NET formation, and the pathophysiology of AF as it relates to the tremendous increase in stroke and transient ischemic attack risk in this patient population [55].

Given the particular enrichment of NETs in thrombi from patients with stroke and AF, it is possible that a larger proportion of their structural integrity is made up of NET chromatin. Older animal model work prior to the discovery of NETs has already demonstrated a possible neuroprotective benefit of DNase 1 treatment to clear extracellular chromatin in stroke models [56]. More recent *ex vivo* studies have also shown better lysis with the cotreatment of retrieved specimens with DNase 1 in addition to tPA [50,51]. Thus, this patient population may be especially responsive to any future adjunctive pharmacotherapies that target NETs.

In addition to facilitating thrombus breakdown through DNase, other options for targeting NETs in this population could involve known inhibitors to NET formation. In particular, the peptidylarginine deiminase inhibitor Cl-amidine has been shown to decrease NET formation in animal models of several inflammatory states [57–59]. The endogenous NET inhibitory factor [60] has been shown to have the same effect through a similar mechanism [58]. Cl-amidine and the NET inhibitory factor have been studied in the context of inflammation and healing after central nervous system injury [61] or stroke [62,63]. While there is some promising work describing their therapeutic potential in acute stroke therapy and primary stroke prevention [63], no work exists to date exploring the role of these agents in the treatment or prevention of stroke in patients with AF.

Another notable finding in the present work includes the significant enrichment of the small Src family tyrosine kinase FGR in thrombi from patients with AF. FGR is known to be involved in regulating platelet activation [64], as well as in neutrophil activation, adhesion, and contact-mediated signaling [65]. While it is not possible from the present work to determine the source of the FGR noted in AF+ thrombi, it is important to recognize the convergence of signaling machinery involved in both platelet and innate immune system signaling. Additionally, the enrichment of the S100A8 and S100A9 proteins in AF thrombi is of special significance, given recent literature highlighting the importance of the calgranulin gene family in modulating

neuroinflammation. Elevated levels of the primarily myeloid cell heterodimer S100A8/A9 have been found to be associated with atherosclerosis progression, oxidative stress, and overall worse prognosis after stroke [66,67]. Specific polymorphisms of the genes have also been found to modulate overall stroke risk, with some variants that are even linked with reduced risk of stroke [66]. Given the ongoing search for biomarkers of stroke risk, diagnosis, treatment response, and eventual prognosis, the elevation of these proteins in AF+ thrombi alongside the presence of activated neutrophils and NETs should be followed up on with more direct assays that explore the relevance of the calgranulin gene family as it either relates to or indeed influences, the differential composition we describe in AF stroke thrombi.

One final notable finding of the present work includes the significant enrichment of eosinophil proteins in AF+ thrombi. Little is known about the role of eosinophils in either stroke or AF beyond associations between peripheral eosinophil counts and stroke severity; a new but small body of work demonstrates the presence of eosinophil extracellular traps in a number of disease processes [68–70], including thrombosis and thrombus stabilization [71]. Our findings of eosinophil enrichment in AF+ thrombi may thus reflect the role of eosinophils in pathologic thrombosis in this population, but more work is needed to explore this possible association.

The present analysis does have some limitations that are important to acknowledge to contextualize our research. First, only 4 of the 18 total detected histone proteins were significantly enriched in AF+ thrombi, although all proteins of the same functional grouping would be expected to cluster together in abundance. This finding may be due to differences in the basal abundance of the 18 histones detected that make certain proteins easier to capture with a smaller sample size. This finding could also be explained by cellular processes within thrombi themselves that render certain histones [72] less likely to be detected by mass spectrometry. While powerful, proteomic profiling only offers a snapshot of a complex process and provides a unique but solitary perspective. Thus, while insights gleaned from abundance data and functional analysis are highly suggestive of NETosis and elevated NET presence in the thrombi retrieved from patients with stroke and AF, it is important to remember that NETosis itself has several distinct triggers. With this in mind, one limitation of our analysis is that our GSEA pipeline necessarily included NET proteomes from patients with autoimmune conditions, though they made up a minority of the input comparison data relative to NET proteomes sourced from healthy patients. Taking all of this into consideration, it is necessary to further examine our findings with either histopathology or more direct mechanistic studies.

Additionally, it is also possible that the proteomic composition of thrombi may be affected by the administration of either i.v. tPA and/or adjunctive IA tPA when applicable. The majority of patients in this study received i.v. and/or IA tPA, and similar proportions of AF+ and AF− patients received tPA, at least partially controlling for this possible confound. Preliminary analysis, though limited by sample size, shows no significant enrichment of marker proteins as a function of tPA administration (Supplementary Figure). However, future analysis using a larger sample size will be better suited to assess this possibility.

Finally, we highlight the limitations inherent to using such broad categories to describe stroke etiology, such as "cardioembolic" [73]. As the present study and others show, there is significant heterogeneity between retrieved stroke thrombi. Indeed, while AF is far and away the most significant cause of "cardioembolic" stroke, conditions such as cardiac amyloidosis, endocarditis, and thrombosis associated with prosthetic heart valves can all be classified in the same category. The current standard for describing stroke pathology distinguishes cases on the basis of thromboembolus origin, but there may come a time in the near future when it is not just more descriptive but also more clinically useful to classify cases on the basis of thromboembolus composition.

The majority of published work treats AF as a stroke etiology, but it may be time to expand current views to consider AF as a comorbidity with a complex relationship with the pathobiology of coagulation. The role of NETs in the context of COVID-19 pathology has been well-established [74], and recent research suggests utility in using subcutaneous DNase to target NETs in ameliorating the COVID disease phenotype in mouse models [75]. Perhaps it is time to expand our view of thrombosis to include NETs in stroke pathology and to explore the possibility of treating or even preventing, stroke in patients with AF using therapeutics that target NETs. The insights gleaned from this analysis are both biologically exciting and clinically useful to the significant patient population with AF. This work contributes to a burgeoning field of omics research on retrieved cerebral thromboemboli by other groups and supplements the large body of largely histopathologic work on this newly accessible biospecimen.

5 | CONCLUSION

We present, for the first time, evidence demonstrating differences in the proteomic composition of retrieved embolic material between patients with stroke with and without AF, suggesting distinct biology involved in stroke thrombus generation with AF. Additionally, we show the enrichment of NETs in thrombi retrieved from patients with AF. A growing body of research is implicating NETs in pathologic thrombosis, especially as it is associated with AF. This finding of a significant NET presence in a patient population at significant risk for stroke may be used to inform future work on not just stroke pathophysiology but also future pharmacotherapies.

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ETHICS STATEMENT

We confirm that the presented research adheres to ethical research methods and adheres to the Strengthening the Reporting of Observational Studies in Epidemiology observational study guidelines. Institutional review board approval was obtained from our institution, and documentation is available upon request. All patient specimens were collected for research with patient consent.

AUTHOR CONTRIBUTIONS

S.M.K.A. conceived the project and wrote the manuscript with significant edits and contributions from all authors. N.R.E., G.S.K., T.B., T.K.M., and M.T.B. acquired consent from all patients included in the study. K.W. assisted with sample preparation and mass spectrometry. S.M.K.A., K.W., and S.C. performed all analysis of data. C.N.M. helped with freezer storage of samples and provided shared his laboratory space for this work. All authors assisted in manuscript editing.

RELATIONSHIP DISCLOSURE

There are no competing interests to disclose.

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SUPPLEMENTARY MATERIAL

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