

MiR-146a Regulates Neutrophil Extracellular Trap Formation That Predicts Adverse Cardiovascular Events in Patients With Atrial Fibrillation

Ana B. Arroyo, Ascensión M. de los Reyes-García, José M. Rivera-Caravaca, Patricia Valledor, Nuria García-Barberá, Vanessa Roldán, Vicente Vicente, Constantino Martínez,* Rocío González-Conejero*

Objective—Atrial fibrillation (AF) patients experience adverse cardiovascular events (ACEs) despite anticoagulant therapy. We reported that rs2431697 of miR-146a, a negative regulator of inflammation, predicts ACEs in patients with AF. The relationship between neutrophil extracellular traps and thrombogenesis is known. Thus, our aim was to evaluate the role of neutrophil extracellular trap compounds as prognostic markers of ACEs in AF and to study whether miR-146a affects NETosis.

Approach and Results—We included 336 steadily anticoagulated AF patients with a median follow-up of 7.9 years (interquartile range, 7.3–8.1) and 127 healthy subjects. The reviewed ACEs included stroke (ischemic/embolic), acute coronary syndrome, acute heart failure, and global or vascular death. We quantified cell-free DNA and NE (neutrophil elastase) at diagnosis. Rs2431697 was genotyped. Neutrophils from human and mice were seeded to analyze shed cell-free DNA and H3cit (citrullinated histone 3) after activation. In human plasmas, higher NE levels (>55.29 ng/mL), but not cell-free DNA, were independently associated with higher risk of all-cause mortality (hazard ratio, 2.24; 95% CI, 1.36–3.68), cardiovascular mortality (hazard ratio, 4.77; 95% CI, 1.11–20.47), and composite cardiovascular events (hazard ratio, 1.84; 95% CI, 1.01–3.76). In patients, NE levels were associated with rs2431697 (TT: 51.82±2.73 versus CC: 40.01±3.05 ng/mL; $P=0.040$). In vitro, both human (TT for rs2431697) and miR-146a^{-/-} mice neutrophils yielded higher levels of cell-free DNA and H3cit than CC or wild-type cells, respectively.

Conclusions—NE activity can provide new ACE prognostic information in AF patients. These findings provide evidence of a potential role of miR-146a in neutrophil extracellular trap generation and cardiovascular risk in AF.

Visual Overview—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2018;38:892-902. DOI: 10.1161/ATVBAHA.117.310597.)

Key Words: atrial fibrillation ■ extracellular trap ■ inflammation ■ microRNA ■ neutrophil

Atrial fibrillation (AF), the most common sustained cardiac arrhythmia, is associated with a 5-fold increase in the risk of stroke and thromboembolism, which results in an increased morbidity and mortality.¹ Classical atherosclerotic risk factors usually underlie such complications, as well as peripheral artery disease or aortic plaque, thus contributing to an increased risk of stroke.² Thus, atherosclerosis and AF are interweaved³ such that vascular disease is included in the CHA₂DS₂-VASc (cardiac failure or dysfunction, hypertension, age ≥75 [doubled], diabetes, stroke [doubled] – vascular disease, age 65–74 y and sex category [female]) score for stroke risk stratification.^{4,5} This score is currently recommended by the American and European guidelines to assess the suitability of thromboprophylaxis with oral anticoagulation in patients with AF. Nonetheless, adverse cardiovascular events (ACEs) in AF-anticoagulated patients have been estimated to be ~4%.^{6,7}

Previous results from our group demonstrated that lower levels of miR-146a, guided by the T variant of rs2431697, are predictors of ACEs in AF patients.⁸ MiR-146a is a negative regulator in innate and inflammatory immune responses through the NF (nuclear factor)-κB pathway, in which its mediators, TLR4 (Toll-like receptor 4), IRAK1 (interleukin-1 receptor-associated kinase 1), and TRAF6 (TNF receptor-associated factor 6), are miR-146a targets.^{9,10} Our findings are in accordance with the new concept of atherosclerosis, which is currently considered a result of a codriven interplay between chronic damage of the intima and the nonresolved inflammation that occurs in response to such injury.^{11,12}

In this work, we investigated the molecular role of miR-146a in the development of ACEs in AF patients. This miRNA is mainly expressed in immune cells, which are one of the main protagonists of thrombogenesis.^{13,14} Thus,

Received on: August 9, 2017; final version accepted on: January 24, 2018.

From the Department of Hematology and Medical Oncology, Morales Meseguer University Hospital, Centro Regional de Hemodonación, University of Murcia, Instituto Murciano de Investigación Biosanitaria Virgen de la Arrixaca (IMIB-Arrixaca), Spain.

*These authors contributed equally to this article.

The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.117.310597/-/DC1>.

Correspondence to Rocío González-Conejero, PhD, or Constantino Martínez, PhD, Centro Regional de Hemodonación, C/ Ronda de Garay S/N, 30003 Murcia, Spain, E-mails roocio.gonzalez@car.m.es or constant@um.es

© 2018 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at <http://atvb.ahajournals.org>

DOI: 10.1161/ATVBAHA.117.310597

Nonstandard Abbreviations and Acronyms	
ACEs	adverse cardiovascular events
AF	atrial fibrillation
cfDNA	cell-free DNA
CHA₂DS₂-VASc	cardiac failure or dysfunction, hypertension, age ≥ 75 [doubled], diabetes, stroke [doubled] – vascular disease, age 65–74 y and sex category [female]
H3cit	citrullinated histone 3
HAS-BLED	hypertension, abnormal renal/liver function, stroke, bleeding history or predisposition, labile INR, elderly, drugs/alcohol concomitantly, age
IL	interleukin
IQR	interquartile range
NE	neutrophil elastase
NETs	neutrophil extracellular traps
TLR4	Toll-like receptor 4
vWF	von Willebrand factor

the infiltration of activated monocytes/neutrophils in arteries mediates an overactivation of the immune system.¹⁵ In addition, findings show that neutrophils shed neutrophil extracellular traps (NETs), which consist of cell-free DNA (cfDNA), histones, NE (neutrophil elastase), and myeloperoxidase that trigger the coagulation and inflammation underlying cardiovascular complications.^{16–18} NET burden has even been described as a predictor of ST-segment resolution and myocardial infarct size,¹⁷ as well as being associated with autoimmune disorders and their thrombotic complications.¹⁹ Indeed, in a large-scale study, NETs have been histologically located in aspirated thrombi of patients with stent thrombosis, supporting their pathophysiological role in this process.²⁰ In this framework, the control of the inflammatory environment in patients with AF is a field for promising, novel therapies.^{15,21}

To date, there is no information on how mir-146a levels can affect the NETosis process. In this work, we intend to study the link between miR-146a and NETosis in relation to the occurrence of ACEs in patients with AF.

Materials and Methods

Patients

We included both healthy subjects and AF patients in this study. Healthy participants were white, unrelated volunteers from our local blood donor center. None of the healthy participants had a documented history of vascular disease, a personal history of thromboembolic/hemorrhagic disease or cancer.

Detailed data from AF patients have been previously reported.⁸ In brief, consecutive patients with permanent or paroxysmal AF who were stable on acenocoumarol treatment (international normalized ratio [INR], 2.0–3.0) were recruited from our outpatient anticoagulation clinic.

Follow-up information was obtained from routine visits to the anticoagulation clinic, from hospital electronic medical records, or when these were unavailable, by telephone interview. The investigators identified, confirmed, and recorded all adverse events.

All subjects who met eligibility criteria were enrolled after providing written informed consent. The study was approved by the Ethical Committee of our institution and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its subsequent amendments.

End Points

As primary ACE end points, we included ischemic stroke, all-cause mortality, cardiovascular mortality, and a combined end point of ACEs (the composite of stroke/transient ischemic attack, acute coronary syndrome, acute heart failure, or cardiovascular death). Ischemic stroke was defined as the sudden onset of a focal neurological deficit in a location consistent with the territory of a major cerebral artery and the result of an obstruction documented by imaging, surgery, or autopsy. Cardiovascular mortality was defined as death caused by sudden death, progressive congestive heart failure and fatal myocardial infarction, or a procedure-related death.

Patients' Samples and Reagents

At inclusion, blood samples were drawn atraumatically and without stasis into tubes preloaded with trisodium citrate (0.011 mol/L). Platelet-poor plasma fractions were obtained by centrifugation at 4°C for 20 minutes at 2200g. Aliquots were stored at –80°C to allow batch analysis. Plasma cfDNA was quantified with Sytox Green (ThermoFisher, Madrid, Spain). Briefly, a standard curve with DNA from salmon sperm (Sigma-Aldrich, Madrid, Spain) was generated for each determination, and plasma samples (10 μ L) were assayed in triplicate. After 15 minutes in the dark, the plate was read at 488 nm in a microplate reader (Biotek Sinergy Ht, Izasa, Barcelona, Spain). The pooled plasma from 40 healthy volunteers was used as a reference.

Levels of NE from plasma were measured with a commercial ELISA (Abcam, Cambridge, UK) following the manufacturer's instructions. All samples were tested in duplicate.

Plasma vWF (von Willebrand factor) antigen levels were assessed in an automated coagulometer ACL Top 3G (Instrumentation Laboratory, Lexington, MA).

hsIL6 (high-sensitivity interleukin-6) levels were assessed by electrochemiluminescence in an automated analyzer (Cobas e601; Roche Diagnostica, Mannheim, Germany).

Genotyping

MIR-146A SNP rs2431697 was genotyped by Taqman analysis, using Taqman SNP Genotyping Assays C_26693319_10 (ThermoFisher). All genotypes were confirmed by sequencing of randomly selected samples.

Isolation of Human Neutrophils

Neutrophils were purified from whole blood of healthy donors, selected by rs2431697 genotype, on Histopaque 1077 (Sigma-Aldrich). The neutrophil pellet was finally resuspended in Hanks balanced salt solution (supplemented with Ca²⁺ and Mg²⁺; ThermoFisher). Cells were counted by means of an automatic cell counter (BioRad, Hercules, CA). Purity was checked by flow cytometry and was always >90% (not shown).

Quantification of MiR-146a Expression

MiR-146a expression in human neutrophils was measured by qRT-PCR (real-time quantitative reverse transcription PCR) using 100 ng of total RNA with MultiScribe Reverse Transcriptase (ThermoFisher) and Premix Ex Taq (Probe qPCR) Master Mix (Takara Bio Company, Condalab, Madrid, Spain). Polymerase chain reaction of miR-146a and U6 (endogenous control) were performed using a Real Time LC480 PCR system (Roche Applied Science, Barcelona, Spain). The 2^{–ΔCt} method was used to calculate the relative abundance of mRNA compared with the expression of the endogenous control.

Mice

MiR-146a^{–/–} mice (The Jackson laboratory, Bar Harbor, ME) were backcrossed for >8 generations in a C57BL/6J background. Inbred wild-type (WT) littermates were used as controls to confront comparative studies. All the mice used in this study were 8 to 12 weeks of age. We included 4 females and 3 males because as far as we know, the miR-146a deficiency in mice affects both sexes equally.

All experimental and other scientific procedures with animals conformed to EU Directive 2010/63/EU and Recommendation 2007/526/EC, enforced in Spanish law under Real Decreto 53/2013.

Isolation of Murine Neutrophils

Primary bone marrow neutrophils were isolated from wild-type and *miR-146a*^{-/-} mice. Briefly, bone marrow cells were collected by flushing the femur with Hanks balanced salt solution using a 26-gauge needle and filtering through a sterile 70- μ m nylon cell strainer. Bone marrow single-cell suspensions were then washed with Hanks balanced salt solution, and primary bone marrow neutrophils were isolated by a negative selection technique (Neutrophil Isolation Kit, Miltenyi Biotec, Barcelona, Spain) and resuspended in Hanks balanced salt solution supplemented with EDTA/Mg²⁺. Neutrophil purity and viability >95% were checked by flow cytometry and propidium iodide, respectively (not shown).

In Vitro Neutrophil Activation

Purified human and mice neutrophils were seeded (600,000 cells/well) in 12-well plates with 0.01% poly-L-lysine (Sigma-Aldrich) for quantification of the free DNA. In addition, 100,000 cells/well were seeded in 2% gelatin-treated coverslips to perform the immunofluorescence study. In both cases, cells were stimulated with 20 nmol/L phorbol-12-myristate-13-acetate (Sigma-Aldrich) in dimethyl sulfoxide. As a negative control of activation, the same number of cells was seeded in each case adding only dimethyl sulfoxide. Cells were incubated for 2 hours (murine neutrophils) or 4 hours (human neutrophils) at 37°C and 5% CO₂.

Quantification of NETosis Markers From In Vitro Activated Neutrophils

DNA released from activated human and mice neutrophils was quantified, after MNaseI treatment (4×10^{-3} U/mL) with Sytox Green (ThermoFisher), and fluorescence was detected as described previously.

H3cit (citrullinated histone 3) expression was quantified by immunofluorescence. For this, coverslips (n=6/sample) were blocked for 1 hour with 2% PBS-BSA and permeabilized with 0.5% PBS-BSA supplemented with 0.1% Triton X-100. Then, cells were incubated for 2 hours with 5 μ g/mL anti-H3cit antibody (for citrulline R2, R8, and R17; Abcam). Alexa Fluor 546 antirabbit secondary antibody (ThermoFisher) was added. Next, coverslips were mounted with ProLong Gold Antifade medium with DAPI (4', 6-diamino-2-phenylindole; ThermoFisher). The preparations were stored at 4°C until analysis.

Image capture was performed using a Leica-DMI6000B fluorescence microscope (Leica, Madrid, Spain). A minimum of 10 fields for each coverslip were captured. The image analysis was performed with ImageJ (<https://imagej.nih.gov/ij/>), and the ratio of H3cit-positive cells/total cells was calculated for each field.

Statistical Analysis

Categorical variables are expressed as frequencies and percentages. Continuous variables were tested for normal distribution by the Kolmogorov-Smirnov test and expressed as the mean $\pm$ SEM or median and interquartile range (IQR), as appropriate. All statistical analyses were performed using parametric test for normally distributed data and nonparametric test for non-normally distributed data.

Differences in the means were assessed by Student *t* test or the Mann-Whitney *U* test, as appropriate, whereas correlations were tested using the statistical Spearman ρ . For multiple comparisons, 1-way ANOVA on ranks with a post hoc test (Bonferroni correction) were used. The χ^2 test was used to compare frequencies. Cox models were performed to determine the association between clinical risk factors, NE, and ACEs. The independent effect of each variable on prognosis was calculated using Cox proportional hazards regression models, incorporating into the multivariate model only those values that showed *P*<0.15 in the univariate analysis. The cutoff point of

each biomarker with the best specificity and sensitivity was chosen using receiver-operating characteristic (ROC) curves.

We performed ROC curves to compare the predictive ability (assessed as C index) of the CHA₂DS₂-VASC score with a modified CHA₂DS₂-VASC score that included the NE. The comparison of both ROC curves was performed by the method of DeLong et al.²² Finally, the increment of the predictive performance was also tested by the integrated discriminatory improvement and the net reclassification improvement.

A *P*<0.05 was accepted as statistically significant. Statistical analyses were performed using SPSS 22.0 for Windows (SPSS, Inc, Chicago, IL), survIDINRI package for R v. 3.3.1 for Windows, and GraphPad Prism 3.3 (GraphPad Software, Inc, La Jolla, CA).

Results

cfDNA Correlates With Age

We performed cfDNA measures in plasma from 127 healthy subjects and 686 AF patients. As shown in Figure 1, we found that there is a significant increase in cfDNA in subjects >75 years of age. To avoid any bias, we only included subjects aged <75 years in later studies (controls: n=100, 33% female, median age: 54 years [IQR, 50–60]; AF patients: n=336 patients, 38% female, median age: 69 years [IQR, 63–72]).

Patients' Clinical, Demographic, and Genetic Characteristics

Demographic and clinical characteristics of the patient cohort are shown in Table 1. The median CHA₂DS₂-VASC score was 3 (IQR, 2–4), and 84.8% of patients had a CHA₂DS₂-VASC score $\geq$ 2. Additionally, all patients were stable on acenocoumarol treatment for at least 6 months before recruitment. The time in therapeutic range 6 months after inclusion was 80% (IQR, 66–100). The median follow-up was 7.9 years (IQR, 7.3–8.1). During this period, we recorded 47 composites of cardiovascular events (14.0%, annual rate: 1.77% per year), 27 ischemic strokes

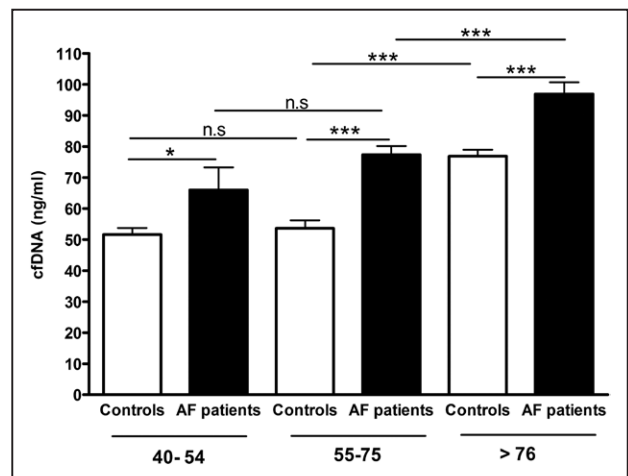


Figure 1. Plasma cell-free DNA (cfDNA) is associated with age in controls and atrial fibrillation (AF) patients. cfDNA was measured using Sytox green as described in the Materials and Methods section. Age ranges: 40 to 54 years (controls: n=53; median age, 50 [interquartile range {IQR}, 46–52] and AF patients: n=20; median age, 52 [IQR, 50–53]); 55 to 75 years (controls: n=47; median age, 60 [IQR: 58–65] and AF patients: n=316, median age, 70 [IQR, 65–73]); and >76 years (controls: n=27, median age, 85 [IQR, 79–89] and AF patients: n=350, median age, 81 [IQR, 78–84]). Data represent mean $\pm$ SEM. **P*<0.05, ****P*<0.001; n.s., not significant.

Table 1. Baseline Clinical Characteristics and rs2431697 Genotype of Patients

	N=336
Demographic	
Male sex, n (%)	206 (61.6)
Age, y, median (IQR)	69 (63–72)
Comorbidities	
Hypertension, n (%)	256 (76.2)
Diabetes mellitus, n (%)	80 (23.8)
Heart failure, n (%)	100 (29.8)
History of stroke/TIA, n (%)	52 (15.5)
Coronary artery disease, n (%)	68 (20.2)
Hepatic impairment, n (%)	5 (1.5)
Renal impairment, n (%)	22 (6.5)
Current smoking habit, n (%)	62 (18.5)
cfDNA, ng/mL, median (IQR)	67.0 (41.3–92.0)
Elastase, ng/mL, median (IQR)	37.8 (26.2–55.3)
Concomitant treatment, n (%)	
Amiodarone	24 (7.1)
Digoxin	45 (13.4)
Calcium antagonist	65 (19.3)
β-Blockers	115 (34.2)
Diuretics	117 (34.8)
ACE inhibitors/ARBs	143 (42.6)
Statins	68 (20.2)
Antiplatelet therapy	53 (15.8)
Scores, median (IQR)	
CHA ₂ DS ₂ -VASc score	3 (2–4)
HAS-BLED score	2 (1–3)
rs2431697 genotype, n (%)	
CC	63 (18.8)
CT	175 (52)
TT	98 (29.2)

ACE indicates angiotensin-converting enzyme; ARB, angiotensin II receptor blockers; CHA₂DS₂-VASc= cardiac failure or dysfunction, hypertension, age ≥75 [doubled], diabetes, stroke [doubled] – vascular disease, age 65–74 y and sex category [female]; HAS-BLED = hypertension, abnormal renal/liver function, stroke, bleeding history or predisposition, labile INR, elderly, drugs/alcohol concomitantly, age; INR, international normalized ratio; IQR, interquartile range; and TIA, transient ischemic attack.

(8.0%, annual rate: 1.02% per year), 72 deaths (21.4%, annual rate: 2.71% per year; 8 of them cardiovascular related), and 44 major bleeding episodes (13.1%, annual rate: 1.66% per year).

Results from rs2431697 genotyping are shown in Table 1. No deviation from the Hardy–Weinberg equilibrium was observed ($P=0.470$).

cfDNA and NE in Plasma Samples

In 100 healthy subjects, we found a mean cfDNA of 54.4 ± 1.95 ng/mL. This value was significantly lower than that found in

the plasma of AF patients (73.7 ± 2.75 ng/mL; $P<0.001$; Figure 1A in the [online-only Data Supplement](#)).

In addition, NE was also higher in the patients' plasma in comparison to the plasma of healthy subjects (48.05 ± 2.07 versus 32.92 ± 2.43 ng/mL, respectively; $P<0.001$; Figure 1B in the [online-only Data Supplement](#)).

Of note, we found a significant positive correlation between cfDNA and NE values in AF patients (Spearman $\rho=0.281$; $P<0.001$; Figure 1C in the [online-only Data Supplement](#)).

cfDNA and NE as Predictors of ACEs

For evaluating the relationship between cfDNA and NE plasma levels with the occurrence of ACEs in AF patients, we used the cutoff points calculated by ROC curves: cfDNA >92 ng/mL and NE >55.29 ng/mL.

Although univariate Cox regression analyses demonstrated that cfDNA levels were not associated with a higher risk of ACEs in patients, this analysis showed a significant increased risk of ACEs for NE levels. According to these analyses, an NE level >55.29 ng/mL was associated with a higher risk of all-cause mortality ($P<0.001$), cardiovascular mortality ($P=0.020$), composite cardiovascular events ($P=0.005$), and ischemic stroke ($P=0.009$; Table 2).

To determine the independent effect of clinical risk factors and NE on the risk of ACEs, we performed subsequent multivariate Cox regression analyses. Thus, NE levels >55.29 ng/mL were independently associated with a higher risk of all-cause mortality (hazard ratio, 2.24; 95% CI, 1.36–3.68), cardiovascular mortality (hazard ratio, 4.77; 95% CI, 1.11–20.47), and the composite of cardiovascular events (hazard ratio, 1.84; 95% CI, 1.01–3.76; Table 3).

For ischemic stroke, only age and previous stroke demonstrated a significant association, whereas NE did not (Table 3).

Kaplan–Meier survival curves clearly showed that higher NE levels were associated with lower time to event for all-cause and cardiovascular mortality and for the composite of cardiovascular events (Figure 2).

Additive Effect of NE Levels to Clinical Risk Scores

We also investigated whether NE levels could improve risk stratification in AF patients. Thus, we included NE in the CHA₂DS₂-VASc score, which is the most widely used risk score for predicting thromboembolic events in AF. The C indexes for the 4 events evaluated were enhanced, and the modified CHA₂DS₂-VASc score was demonstrated to significantly predict all-cause mortality (0.628; 95% CI, 0.574–0.680; $P=0.001$), cardiovascular events (0.675; 95% CI, 0.622–0.725; $P<0.001$), and ischemic stroke (0.716; 95% CI, 0.665–0.764; $P<0.001$; Table 4; Figure 3). However, the comparison of ROC curves did not show a significant higher predictive ability of the CHA₂DS₂-VASc+NE versus the original CHA₂DS₂-VASc (Table 4).

Additionally, we tested the sensitivity and reclassification of using the integrated discriminatory improvement and net reclassification improvement analyses. Overall, the CHA₂DS₂-VASc+NE score showed nonsignificant higher sensitivity and positive reclassification for cardiovascular mortality, cardiovascular events, and ischemic stroke compared with the original CHA₂DS₂-VASc score. Nevertheless, we

Table 2. Univariate Cox Regression Analyses

	All-Cause Mortality		Cardiovascular Mortality		Composite CV Events		Ischemic Stroke	
	Univariate Analysis, HR (95% CI)	P Value	Univariate analysis, HR (95% CI)	P Value	Univariate analysis, HR (95% CI)	P Value	Univariate analysis, HR (95% CI)	P Value
Age (<75 y)	1.04 (1.00–1.09)	0.047	1.05 (0.92–1.19)	0.493	1.04 (0.99–1.10)	0.111	1.16 (1.05–1.29)	0.003
Male sex	1.80 (1.07–3.04)	0.028	1.07 (0.26–4.49)	0.924	0.94 (0.52–1.68)	0.833	0.93 (0.43–2.01)	0.860
Hypertension	0.93 (0.55–1.57)	0.778	0.98 (0.20–4.86)	0.980	1.06 (0.54–2.07)	0.876	1.86 (0.64–5.38)	0.252
Diabetes mellitus	1.78 (1.05–2.80)	0.031	2.00 (0.48–8.40)	0.340	1.46 (0.78–2.73)	0.235	1.00 (0.40–2.47)	0.994
Previous stroke/TIA	2.78 (1.68–4.60)	<0.001	3.59 (0.86–15.02)	0.081	5.27 (2.94–9.44)	<0.001	9.20 (4.28–19.73)	<0.001
Heart failure	1.53 (0.95–2.47)	0.080	1.52 (0.36–6.35)	0.569	1.07 (0.57–2.00)	0.844	0.58 (0.22–1.52)	0.264
Coronary artery disease	1.57 (0.94–2.63)	0.088	1.40 (0.28–6.93)	0.682	1.25 (0.63–2.45)	0.525	1.18 (0.48–2.93)	0.716
Baseline tobacco use	1.87 (1.11–3.13)	0.018	2.86 (0.68–11.95)	0.151	2.32 (1.26–4.28)	0.007	1.77 (0.75–4.18)	0.196
cfDNA >92 ng/mL	1.45 (0.88–2.37)	0.145	0.43 (0.53–3.50)	0.431	1.46 (0.79–2.70)	0.225	1.84 (0.84–4.02)	0.126
Elastase >55.29 ng/mL	1.92 (1.52–2.41)	<0.001	5.49 (1.31–23.01)	0.020	2.29 (1.28–4.10)	0.005	2.76 (1.29–5.89)	0.009

cfDNA indicates cell-free DNA; CI, confidence interval; CV, cardiovascular; HR, hazard ratio; and TIA, transient ischemic attack.

found significant higher sensitivity (integrated discriminatory improvement=0.024; $P<0.001$) and positive reclassification (net reclassification improvement=0.252; $P=0.020$) of $\text{CHA}_2\text{DS}_2\text{-VASc}$ score for all-cause mortality when NE was added to the score (Table I in the [online-only Data Supplement](#)).

Endothelial Damage and Inflammation Markers

AF patients with high NE plasma levels (>55.29 ng/mL) also had significantly higher vWF antigen (median, 168.0 [IQR, 129.9–229.0] versus 149.8 [IQR, 121.8–211.6] UI/dL; $P=0.041$) and hsIL6 levels (median, 3.61 [IQR, 2.05–7.86] versus 2.51 [IQR, 1.50–4.30] pg/mL; $P<0.001$) than AF patients with lower NE plasma levels (<55.29 ng/mL; Table 5).

Relationship Between rs2431697 and NE in Healthy Subjects and in AF Patients

We did not find a significant association between the rs2431697 genotype and NE levels in healthy subjects (Figure IIA in the

[online-only Data Supplement](#)). In AF patients, NE levels of CC homozygous ($n=63$) were significantly lower than those of T allele carriers ($n=252$; 40.01 ± 3.05 versus 51.82 ± 2.73 ng/mL, respectively; $P=0.040$; Figure IIB in the [online-only Data Supplement](#)). In addition, among patients with the highest NE values (>55.29 ng/mL), we found a frequency almost 2-fold higher in subjects bearing the T allele (29%) than for C homozygotes (13%; $P=0.008$; Table 5).

miR-146a Genotype Conditions NET Formation

Human neutrophils from 14 healthy donors were isolated (TT=7 and CC=7). As expected, miR-146a expression levels in neutrophils from T allele carriers were $\approx 40\%$ lower than those from homozygous C carriers, although these results were not statistically significant, probably because of the small sample size (Figure III in the [online-only Data Supplement](#)). To investigate whether rs2431697 impacts NETosis, we further activated neutrophils with phorbol-12-myristate-13-acetate. The results of

Table 3. Multivariate Cox Regression Analyses

	All-Cause Mortality		Cardiovascular Mortality		Composite CV events		Ischemic stroke	
	Multivariate Analysis, HR (95% CI)	P Value	Multivariate Analysis, HR (95% CI)	P Value	Multivariate Analysis, HR (95% CI)	P Value	Multivariate Analysis, HR (95% CI)	P Value
Age (<75 y)	1.03 (0.99–1.08)	0.133	...	...	1.02 (0.97–1.08)	0.396	1.13 (1.03–1.25)	0.014
Male sex	1.71 (0.97–3.02)	0.063	...	...	...	...	...	...
Hypertension	...	...	...	...	...	...	...	...
Diabetes mellitus	1.27 (0.76–2.12)	0.356	...	...	...	...	...	...
Previous stroke/TIA	2.13 (1.26–3.59)	0.005	2.70 (0.63–11.59)	0.180	4.41 (2.42–8.03)	<0.001	7.49 (3.42–16.39)	<0.001
Heart failure	1.59 (0.97–2.59)	0.066	...	...	...	...	...	...
Coronary artery disease	1.19 (0.69–2.06)	0.536	...	...	...	...	...	...
Baseline tobacco use	1.40 (0.78–2.47)	0.259	...	...	2.18 (1.17–4.04)	0.014	...	...
cfDNA >92 ng/mL	1.00 (0.60–1.69)	0.988	...	...	...	...	1.44 (0.65–3.17)	0.368
Elastase >55.29 ng/mL	2.24 (1.36–3.68)	0.002	4.77 (1.11–20.47)	0.035	1.84 (1.01–3.76)	0.048	1.60 (0.72–3.53)	0.251

cfDNA indicates cell-free DNA; CI, confidence interval; CV, cardiovascular; HR, hazard ratio; and TIA, transient ischemic attack.

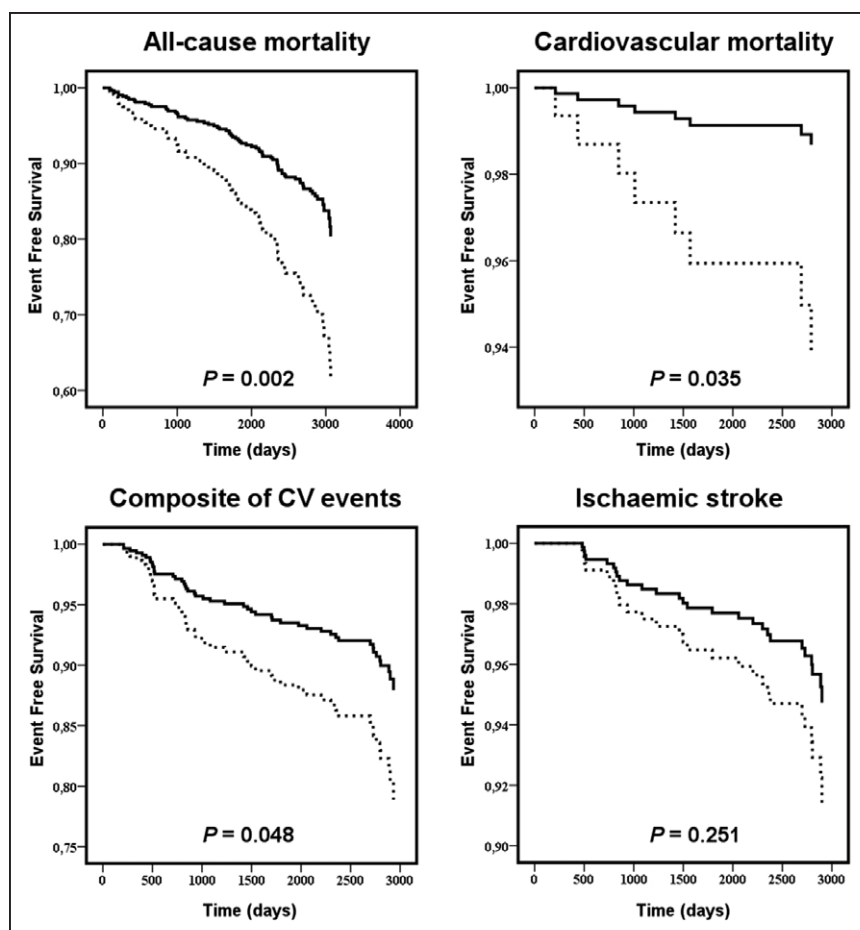


Figure 2. Event-free survival for adverse events according to elastase levels. Solid line represents patients with elastase ≤ 55.29 ng/mL, and dotted line represents patients with elastase > 55.29 ng/mL.

DNA shed from seeded neutrophils are shown in Figure 4A. After activation with phorbol-12-myristate-13-acetate, neutrophils of TT homozygous subjects had a higher relative increase in DNA than CC subjects (Figure 4A). Next, we quantified the expression of H3cit by indirect immunofluorescence as a specific marker

of NETosis. Again, we found a relationship between rs2431697 genotype and H3cit expression. As shown (Figure 3B through 3D), the ratio of H3cit-positive cells/total cells was markedly higher in neutrophils from TT subjects in comparison to CC subjects ($5.15 \pm 0.51\%$ versus $1.21 \pm 0.15\%$, respectively; $P < 0.001$).

Table 4. Comparison of the ROC Curves of the CHA₂DS₂-VASC Score and Modified CHA₂DS₂-VASC Score With NE

	C Index	95% CI	P Value	z Statistics*	P Value*
All-cause mortality					
CHA ₂ DS ₂ -VASC score	0.596	0.542–0.649	0.014	1.637	0.102
CHA ₂ DS ₂ -VASC+NE	0.628	0.574–0.680	0.001		
Cardiovascular mortality					
CHA ₂ DS ₂ -VASC score	0.587	0.532–0.640	0.442	1.340	0.180
CHA ₂ DS ₂ -VASC+NE	0.657	0.603–0.707	0.221		
Composite of cardiovascular events					
CHA ₂ DS ₂ -VASC score	0.655	0.601–0.706	<0.001	0.838	0.402
CHA ₂ DS ₂ -VASC+NE	0.675	0.622–0.725	<0.001		
Ischemic stroke					
CHA ₂ DS ₂ -VASC score	0.687	0.635–0.736	<0.001	0.896	0.370
CHA ₂ DS ₂ -VASC+NE	0.716	0.665–0.764	<0.001		

CHA₂DS₂-VASC, cardiac failure or dysfunction, hypertension, age ≥ 75 [doubled], diabetes, stroke [doubled] – vascular disease, age 65–74 y and sex category [female]; CI, confidence interval; NE, neutrophil elastase; and ROC, receiver-operating characteristic.

*For C index comparison.

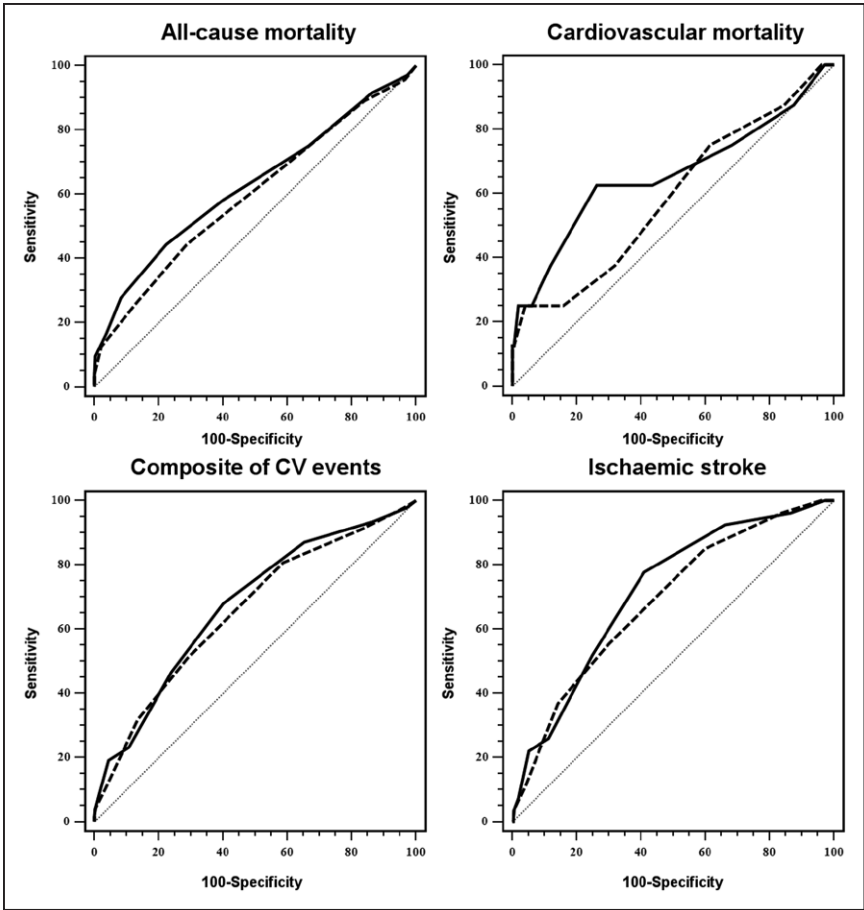


Figure 3. Receiver-operating characteristic curves for original and modified CHA₂DS₂-VASc (cardiac failure or dysfunction, hypertension, age ≥75 [doubled], diabetes, stroke [doubled] – vascular disease, age 65–74 y and sex category [female]) score (elastase >55.29 ng/mL). Solid line represents modified CHA₂DS₂-VASc score, dashed line represents original CHA₂DS₂-VASc score, and dotted gray line represents reference. CV indicates cardiovascular.

Absence of MiR-146a in Murine Neutrophils Increases NET Formation

After activation with phorbol-12-myristate-13-acetate, miR-146a^{-/-} neutrophils (n=7/genotype) had a higher relative increase in DNA than WT mice (3.67±0.34 ng/μL versus 2.84±0.28 ng/μL; *P*<0.05; Figure 5A). Next, we quantified the expression of H3c by indirect immunofluorescence as a specific marker of NETosis. The ratio of H3c-positive cells/total cells were markedly higher in neutrophils from miR-146a^{-/-} mice in comparison to their WT littermates (5.14±0.33% versus 3.21±0.26%, respectively; *P*<0.001; Figure 5B through 5D).

Discussion

Our results revealed that NETosis is a new component of the inflammatory background and atherothrombosis underlying AF.^{23,24} These findings suggest that high NE levels could even delimit an inflammatory burden before arrhythmia. Thus, we

found that patients with higher vWF and interleukin (IL)-6 values also had the highest NE plasma levels. If one of these elements precedes the others, it is difficult to define, but all of them count for the nexus between inflammation and AF argued in recent years as a result of observational and ex vivo clinical studies.²³ For its part, cfDNA levels in patients with AF proved not to be associated with ACEs. Notwithstanding, cfDNA is a nonspecific NETosis marker as it is mainly derived from non-neutrophil dead cells,²⁵ whereas NE quantification is currently considered one of the most specific, objective, and quantitative assay to monitor NETosis.²⁶

Studies have shown that, besides cytokines, NETosis markers are subrogated proinflammatory and prognostic factors in several cardiac pathologies.^{16,17,27–30} In fact, NE has been shown to be elevated in human atherosclerotic plaque shoulders.³¹ More recently, NET burden has been associated with the resolution of the ST-segment and myocardial infarct size.¹⁷ Mechanistically, it has been shown that high levels of

Table 5. vWF and IL6 Levels and rs2431697 Frequencies According to NE Levels in AF Patients

	vWF, UI/dL	<i>P</i> Value*	IL6, pg/mL	<i>P</i> Value*	rs2431697		
	Median (IQR)		Median (IQR)		CC, n (%)	CT+TT, n (%)	<i>P</i> Value†
NE <4Q (n=234)	149.8 (121.8–211.6)	0.041	2.51 (1.50–4.30)	<0.001	55 (87.3)	179 (71.0)	0.008
NE >4Q (n=81)	168.0 (129.9–229.0)		3.61 (2.05–7.86)		8 (12.7)	73 (29.0)	

4Q indicates 4th quartile; IL6, interleukin-6; IQR, interquartile range; NE, neutrophil elastase; and vWF, von Willebrand factor.

*Mann–Whitney *U* test.

†χ² test.

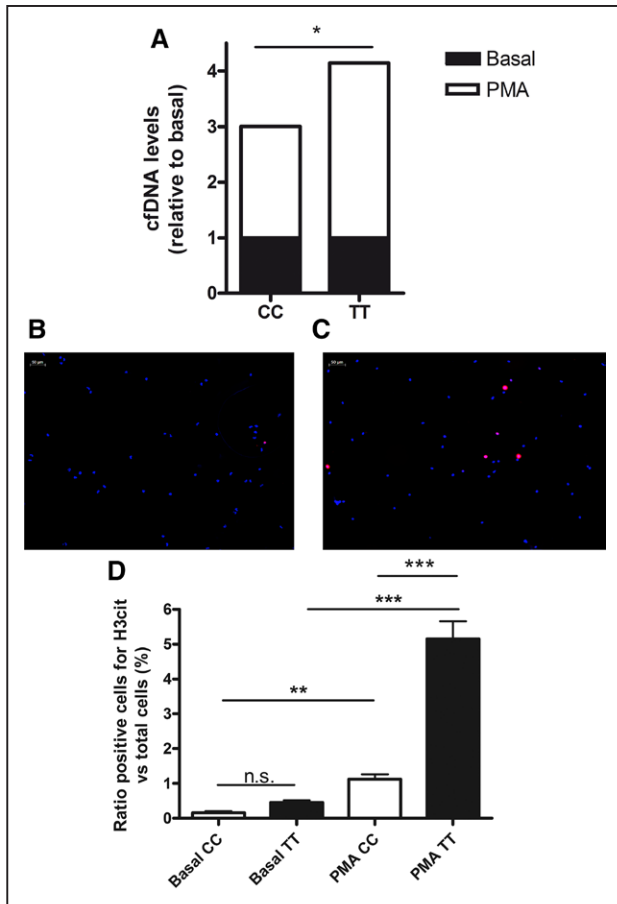


Figure 4. MiR-146a partial deficiency is associated with the formation of neutrophil extracellular traps in vitro. **A**, Quantification by Sytox Green of cell-free DNA (cfDNA) after exposure to PMA (phorbol-12-myristate-13-acetate; 20 nmol/L) in neutrophils from healthy volunteers CC (n=7) and TT (n=7) for rs2431697. Representative images of neutrophil treated with PMA (blue: DAPI [4', 6-diamino-2-phenylindole] and red: H3cit [citruillinated histone 3]), **(B)** CC subjects, and **(C)** TT subjects. All micrographs have a $\times 10$ magnification. **D**, Quantification by indirect immunofluorescence of H3cit levels after exposure to PMA (20 nmol/L) or in basal conditions in neutrophils from donors with genotype TT vs CC genotype (n=7 subjects/group). Data represent mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

NE regulate the IL-1 β release from the endothelium, which is related to inflammatory cardiovascular disease.³² Here, we show that NET components are also new prognostic markers for ACEs in AF patients. Therefore, the combination of high NE levels with other comorbidities often experienced by AF patients, such as coronary microvascular dysfunction of hypertension, diabetes mellitus, chronic heart failure, and left ventricular hypertrophy,³³ might help to reach an inflammatory threshold that leads to ACEs. It is well known that not all AF patients have the same risk for an ACE, not even those patients with the same CHA₂DS₂-VASc score. Moreover, the pathogenic mechanisms of the end points considered in this study also differ between patients. Interestingly, patients who experience stroke (n=27), generally a thromboembolic end point, had higher NE values than the other patients (41.12 [IQR, 26.8–81.8] versus 37.73 [IQR, 26.1–54.3]; $P = 0.340$), although these differences were not statistically

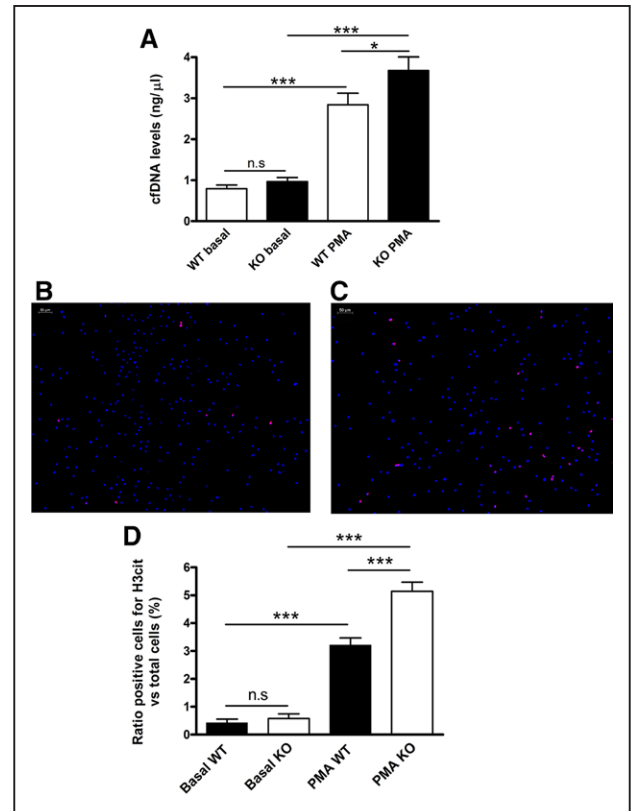


Figure 5. MiR-146a ablation is associated with neutrophil extracellular trap formation in vitro. **A**, Quantification by Sytox Green of cell-free DNA (cfDNA) after exposure to PMA (phorbol-12-myristate-13-acetate; 20 nmol/L) in wild type murine neutrophils or miR-146a KO (n=7/group). Representative images of murine neutrophils treated with PMA (blue: DAPI [4', 6-diamino-2-phenylindole] and red: H3cit [citruillinated histone 3]), **(B)** wild type (WT), and **(C)** miR-146a knockout (KO) mice. All micrographs have a $\times 10$ magnification. **D**, Quantification by indirect immunofluorescence of H3cit levels after exposure to PMA or in basal conditions in murine neutrophils (n=7/group). Data represent mean $\pm$ SEM. * $P < 0.05$, *** $P < 0.001$.

significant. The low number of patients with stroke in the study population did not allow us to draw any conclusion about this issue, which should, therefore, be explored further in future studies.

The role of biomarkers in the prediction of ACEs has been extensively investigated and their value in the everyday real-world clinical evaluation of AF patients is debated.^{34,35} Thus, the clinical use of adding a biomarker to clinical scores remains limited given the marginal net benefit, especially after long-term follow-up.³⁶ In the present study, after adding NE, the predictive ability for all-cause mortality, cardiovascular mortality, cardiovascular events, and ischemic stroke was slightly increased, although it was not significantly different from the original CHA₂DS₂-VASc score. Thus, adding NE as a biomarker did not improve this clinical risk score. In a recent report, we showed that the addition of other biomarkers enhanced the predictive value of CHA₂DS₂-VASc and HAS-BLED (hypertension, abnormal renal/liver function, stroke, bleeding history or predisposition, labile INR, elderly, drugs/alcohol concomitantly, age) score, but the addition provided only a modest overall improvement, and marginal added

predictive advantage over the original scores.³⁷ However, the present study has demonstrated that NE was independently associated with a higher risk of cardiovascular events, cardiovascular mortality, and all-cause mortality in these patients. This fact is clinically relevant, and because this study included a cohort of young AF patients (<75 years), future studies are needed to further investigate this association.

MiRNAs have been recognized as new components of the pathological processes of cardiovascular diseases.^{38–40} In particular, our group demonstrated the association of miR-146a with a higher risk of ACEs in AF patients bearing the T variant of rs2431697.⁸ However, to date, a relationship between miRNAs and NETosis has not been described. Here, we give further molecular clues to the association between rs2431697 and the occurrence of ACEs. Thus, our *in vitro* results show that the inflammatory stimulus delimitates differences in the susceptibility to NETosis driven by rs2431697. In this manner, we suggest that rs2431697 may be associated with NE levels in patients with AF, thus promoting a higher rate of ACE occurrence.

Our results from WT and miR-146a^{−/−} murine neutrophils point to a causal relationship between miR-146a and NETosis. However, the mechanism behind the action of miR-146a is still unknown. MiRNAs work by binding to mRNA and inhibiting their translation. It has been described that NET production is independent of the expression of *de novo* genes; in other words, neutrophils already contain all the necessary factors for the formation of NETs when they leave the bone marrow.⁴¹ Therefore, miR-146a must play its role before cell activation. It has been demonstrated that a lack of miR-146a targets in mice, such as TLR4, prevents NET formation.⁴² Moreover, it has been shown in a mouse model that NET components increase with aging.³⁰ Therefore, we speculate that lower levels of miR-146a because of rs2431697 will also contribute to NETosis by a mechanism that is yet to be identified. Consequently, this genotype may contribute to age-related inflammation leading to atherosclerosis and the progression of cardiovascular diseases.⁴³

Several preclinical therapies for preventing atherosclerosis and its harmful effects are already underway. Some of these therapies are focused on reducing as much of the NETosis process as possible, and they consist of pharmacological or genetic tools that degrade DNA or inhibit histone citrullination or of enzymes that interfere with the respiratory chain, leading to ROS clearance or enhancing NET clearance. All these strategies are meant to cause a decrease in NET release and to control their detrimental consequences.^{44,45} Our results suggest that miR-146a could play a valuable role in complementing these therapies as its increase has been shown to be beneficial in inhibiting atherosclerosis.^{46,47} Further studies are needed to assess the role of NETs and miR-146a in patients with AF and to test their therapeutic usefulness in this disease and other inflammatory diseases.

Limitations of the Study

There are some limitations regarding this study that we must acknowledge. First, it was performed in a single center, and

it composed of a white-based population. Second, all AF patients had good anticoagulation control during the previous 6 months after entry, to guarantee that the impact of the biomarkers was not related to poor anticoagulation control. Additionally, we selected patients who were anticoagulated according to their CHA₂DS₂-VASc score. Therefore, our results should only be extrapolated to clinically similar patients. Whether NET compounds are prognostic markers of ACEs in AF patients without comorbidities is an interesting issue not explored in our study but that deserves further investigation.

We have measured the NET formation in samples at diagnosis, and no other determinations have been performed throughout the 7.9-year follow-up. How the predictor factors for ACEs identified here evolve throughout the follow-up period has not been determined. Because comorbidities change over time, we expect that biomarkers will also change. However, because our aim was to find factors with the ability to predict ACEs during a long follow-up, we remain confident in our results.

In recent years, the neutrophil/lymphocyte ratio has been associated with inflammation and worse outcomes in patients with cardiovascular diseases. It would have been timely to analyze the association between neutrophil/lymphocyte ratio and ACEs and the rs2431697 genotype in our series. However, 8 years ago, analytic data were not computerized in our hospital, and we have been unable to perform such analyses. In addition, we cannot rule out that other causes than NETosis such as neutrophil degranulation or a higher neutrophil count may also be behind the high levels of plasma NE in patients with ACEs. Additional studies are needed to establish the relationship between the rs2431697 genotype and the risk of ACEs in AF patients.

Acknowledgments

We are extremely grateful to Dr Ginés Luengo-Gil for his assistance with data analysis, to Elisa Tornero for her help recruiting elder donors, to all of them, and to all the healthy volunteers who were involved in the *in vitro* studies.

Sources of Funding

This work was supported by research grants from Instituto de Salud Carlos III, Fondo Europeo de Desarrollo Regional (P14/00253), and Fundación Séneca 19873/GERM/15. A.B. Arroyo has a research fellowship from Universidad de Murcia. J.M. Rivera-Caravaca is supported by Instituto Murciano de Investigación Biosanitaria (IMIB16/AP/01/06).

Disclosures

None.

References

1. Chugh SS, Havmoeller R, Narayanan K, Singh D, Rienstra M, Benjamin EJ, Gillum RF, Kim YH, McAnulty JH Jr, Zheng ZJ, Forouzanfar MH, Naghavi M, Mensah GA, Ezzati M, Murray CJ. Worldwide epidemiology of atrial fibrillation: a Global Burden of Disease 2010 Study. *Circulation*. 2014;129:837–847. doi: 10.1161/CIRCULATIONAHA.113.005119.
2. Violi F, Soliman EZ, Pignatelli P, Pastori D. Atrial fibrillation and myocardial infarction: a systematic review and appraisal of pathophysiologic mechanisms. *J Am Heart Assoc*. 2016;5:e003347. doi: 10.1161/JAHA.116.003347.

3. Willeit K, Kiechl S. Atherosclerosis and atrial fibrillation—two closely intertwined diseases. *Atherosclerosis*. 2014;233:679–681. doi: 10.1016/j.atherosclerosis.2013.11.082.
4. January CT, Wann LS, Alpert JS, et al; American College of Cardiology/American Heart Association Task Force on Practice Guidelines. 2014 AHA/ACC/HRS guideline for the management of patients with atrial fibrillation: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines and the Heart Rhythm Society. *J Am Coll Cardiol*. 2014;64:e1–e76. doi: 10.1016/j.jacc.2014.03.022.
5. Kirchhof P, Benussi S, Kotecha D, et al. 2016 ESC guidelines for the management of atrial fibrillation developed in collaboration with EACTS. *Kardiol Pol*. 2016;74:1359–1469. doi: 10.5603/KP.2016.0172.
6. Hart RG, Pearce LA, Aguilar MI. Meta-analysis: antithrombotic therapy to prevent stroke in patients who have nonvalvular atrial fibrillation. *Ann Intern Med*. 2007;146:857–867.
7. Lip GY, Nieuwlaar R, Pisters R, Lane DA, Crijns HJ. Refining clinical risk stratification for predicting stroke and thromboembolism in atrial fibrillation using a novel risk factor-based approach: the euro heart survey on atrial fibrillation. *Chest*. 2010;137:263–272. doi: 10.1378/chest.09-1584.
8. Roldán V, Arroyo AB, Salloum-Asfar S, Manzano-Fernández S, García-Barberá N, Marín F, Vicente V, González-Conejero R, Martínez C. Prognostic role of MIR146A polymorphisms for cardiovascular events in atrial fibrillation. *Thromb Haemost*. 2014;112:781–788. doi: 10.1160/TH14-01-0092.
9. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc Natl Acad Sci USA*. 2006;103:12481–12486. doi: 10.1073/pnas.0605298103.
10. Yang K, He YS, Wang XQ, Lu L, Chen QJ, Liu J, Sun Z, Shen WF. MiR-146a inhibits oxidized low-density lipoprotein-induced lipid accumulation and inflammatory response via targeting toll-like receptor 4. *FEBS Lett*. 2011;585:854–860. doi: 10.1016/j.febslet.2011.02.009.
11. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med*. 1999;340:115–126. doi: 10.1056/NEJM199901143400207.
12. Santovito D, Weber C. Atherosclerosis revisited from a clinical perspective: still an inflammatory disease? *Thromb Haemost*. 2017;117:231–237. doi: 10.1160/TH16-10-0770.
13. Boldin MP, Taganov KD, Rao DS, Yang L, Zhao JL, Kalwani M, Garcia-Flores Y, Luong M, Devrekanli A, Xu J, Sun G, Tay J, Linsley PS, Baltimore D. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. *J Exp Med*. 2011;208:1189–1201. doi: 10.1084/jem.20101823.
14. Swystun LL, Liaw PC. The role of leukocytes in thrombosis. *Blood*. 2016;128:753–762. doi: 10.1182/blood-2016-05-718114.
15. Drechsler M, Döring Y, Megens RT, Soehnlein O. Neutrophilic granulocytes - promiscuous accelerators of atherosclerosis. *Thromb Haemost*. 2011;106:839–848. doi: 10.1160/TH11-07-0501.
16. Borisoff JJ, Joosen IA, Versteylen MO, Brill A, Fuchs TA, Savchenko AS, Gallant M, Martinot K, Ten Cate H, Hofstra L, Crijns HJ, Wagner DD, Kietzelaer BLJH. Elevated levels of circulating DNA and chromatin are independently associated with severe coronary atherosclerosis and a prothrombotic state. *Arterioscler Thromb Vasc Biol*. 2013;33:2032–2040. doi: 10.1161/ATVBAHA.113.301627.
17. Mangold A, Alias S, Scherz T, et al. Coronary neutrophil extracellular trap burden and deoxyribonuclease activity in ST-elevation acute coronary syndrome are predictors of ST-segment resolution and infarct size. *Circ Res*. 2015;116:1182–1192. doi: 10.1161/CIRCRESAHA.116.304944.
18. Döring Y, Soehnlein O, Weber C. Neutrophil extracellular traps in atherosclerosis and atherothrombosis. *Circ Res*. 2017;120:736–743. doi: 10.1161/CIRCRESAHA.116.309692.
19. Pérez-Sánchez C, Ruiz-Limón P, Aguirre MA, et al. Diagnostic potential of NETosis-derived products for disease activity, atherosclerosis and therapeutic effectiveness in Rheumatoid arthritis patients. *J Autoimmun*. 2017;82:31–40. doi: 10.1016/j.jaut.2017.04.007.
20. Riegger J, Byrne RA, Joner M, et al; Prevention of Late Stent Thrombosis by an Interdisciplinary Global European Effort (PRESTIGE) Investigators. Histopathological evaluation of thrombus in patients presenting with stent thrombosis. A multicenter European study: a report of the prevention of late stent thrombosis by an interdisciplinary global European effort consortium. *Eur Heart J*. 2016;37:1538–1549. doi: 10.1093/eurheartj/ehv419.
21. Murphy AJ, Tall AR. Disordered haematopoiesis and athero-thrombosis. *Eur Heart J*. 2016;37:1113–1121. doi: 10.1093/eurheartj/ehv718.
22. DeLong ER, DeLong DM, Clarke-Pearson DL. Comparing the areas under two or more correlated receiver operating characteristic curves: a nonparametric approach. *Biometrics*. 1988;44:837–845.
23. Hu YF, Chen YJ, Lin YJ, Chen SA. Inflammation and the pathogenesis of atrial fibrillation. *Nat Rev Cardiol*. 2015;12:230–243. doi: 10.1038/nrcardio.2015.2.
24. Depta JP, Bhatt DL. Atherothrombosis and atrial fibrillation: important and often overlapping clinical syndromes. *Thromb Haemost*. 2010;104:657–663. doi: 10.1160/TH10-05-0332.
25. Bronkhorst AJ, Aucamp J, Pretorius PJ. Cell-free DNA: preanalytical variables. *Clin Chim Acta*. 2015;450:243–253. doi: 10.1016/j.cca.2015.08.028.
26. Masuda S, Nakazawa D, Shida H, Miyoshi A, Kusunoki Y, Tomaru U, Ishizu A. NETosis markers: quest for specific, objective, and quantitative markers. *Clin Chim Acta*. 2016;459:89–93. doi: 10.1016/j.cca.2016.05.029.
27. Jones DP, True HD, Patel J. Leukocyte trafficking in cardiovascular disease: insights from experimental models. *Mediators Inflamm*. 2017;2017:9746169. doi: 10.1155/2017/9746169.
28. Rudolph V, Andrié RP, Rudolph TK, et al. Myeloperoxidase acts as a profibrotic mediator of atrial fibrillation. *Nat Med*. 2010;16:470–474. doi: 10.1038/nm.2124.
29. Friedrichs K, Adam M, Remane L, Mollenhauer M, Rudolph V, Rudolph TK, Andrié RP, Stöckigt F, Schrickel JW, Ravakes T, Deuschl F, Nickenig G, Willems S, Baldus S, Klinka A. Induction of atrial fibrillation by neutrophils critically depends on CD11b/CD18 integrins. *PLoS One*. 2014;9:e89307. doi: 10.1371/journal.pone.0089307.
30. Martinot K, Witsch T, Erpenbeck L, Savchenko A, Hayashi H, Cherpokova D, Gallant M, Mauler M, Cifuni SM, Wagner DD. Peptidylarginine deiminase 4 promotes age-related organ fibrosis. *J Exp Med*. 2017;214:439–458. doi: 10.1084/jem.20160530.
31. Dollery CM, Owen CA, Sukhova GK, Krettek A, Shapiro SD, Libby P. Neutrophil elastase in human atherosclerotic plaques: production by macrophages. *Circulation*. 2003;107:2829–2836. doi: 10.1161/01.CIR.0000072792.65250.4A.
32. Alfaidi M, Wilson H, Daigneault M, Burnett A, Ridger V, Chamberlain J, Francis S. Neutrophil elastase promotes interleukin-1 β secretion from human coronary endothelium. *J Biol Chem*. 2015;290:24067–24078. doi: 10.1074/jbc.M115.659029.
33. Camici PG, Crea F. Coronary microvascular dysfunction. *N Engl J Med*. 2007;356:830–840. doi: 10.1056/NEJMra061889.
34. Aulin J, Siegbahn A, Hijazi Z, Ezekowitz MD, Andersson U, Connolly SJ, Huber K, Reilly PA, Wallentin L, Oldgren J. Interleukin-6 and C-reactive protein and risk for death and cardiovascular events in patients with atrial fibrillation. *Am Heart J*. 2015;170:1151–1160. doi: 10.1016/j.ahj.2015.09.018.
35. Hijazi Z, Oldgren J, Siegbahn A, Wallentin L. Application of biomarkers for risk stratification in patients with atrial fibrillation. *Clin Chem*. 2017;63:152–164. doi: 10.1373/clinchem.2016.255182.
36. García-Fernández A, Roldán V, Rivera-Caravaca JM, Hernández-Romero D, Valdés M, Vicente V, Lip GY, Marín F. Does von Willebrand factor improve the predictive ability of current risk stratification scores in patients with atrial fibrillation? *Sci Rep*. 2017;7:41565. doi: 10.1038/srep41565.
37. Roldán V, Rivera-Caravaca JM, Shantsila A, García-Fernández A, Esteve-Pastor MA, Vilchez JA, Romera M, Valdés M, Vicente V, Marín F, Lip GYH. Enhancing the ‘real world’ prediction of cardiovascular events and major bleeding with the CHA2DS2-VASc and HAS-BLED scores using multiple biomarkers. *Ann Med*. 2018;50:26–34. doi: 10.1080/07853890.2017.1378429.
38. Small EM, Frost RJ, Olson EN. MicroRNAs add a new dimension to cardiovascular disease. *Circulation*. 2010;121:1022–1032. doi: 10.1161/CIRCULATIONAHA.109.889048.
39. van Rooij E. The art of microRNA research. *Circ Res*. 2011;108:219–234. doi: 10.1161/CIRCRESAHA.110.227496.
40. Thum T, Condorelli G. Long noncoding RNAs and microRNAs in cardiovascular pathophysiology. *Circ Res*. 2015;116:751–762. doi: 10.1161/CIRCRESAHA.116.303549.
41. Sollberger G, Amulic B, Zychlinsky A. Neutrophil extracellular trap formation is independent of de novo gene expression. *PLoS One*. 2016;11:e0157454. doi: 10.1371/journal.pone.0157454.
42. Huang H, Tohme S, Al-Khafaji AB, Tai S, Loughran P, Chen L, Wang S, Kim J, Billiar T, Wang Y, Tsung A. Damage-associated molecular pattern-activated neutrophil extracellular trap exacerbates sterile

- inflammatory liver injury. *Hepatology*. 2015;62:600–614. doi: 10.1002/hep.27841.
43. Fougere B, Boulanger E, Nourhashemi F, Guyonnet S, Cesari M. Chronic inflammation: accelerator of biological aging. *J Gerontol A Biol Sci Med Sci*. 2017;72:1218–1225. doi: 10.1093/gerona/glw240.
 44. Lewis HD, Liddle J, Coote JE, et al. Inhibition of PAD4 activity is sufficient to disrupt mouse and human NET formation. *Nat Chem Biol*. 2015;11:189–191. doi: 10.1038/nchembio.1735.
 45. Stakos DA, Kambas K, Konstantinidis T, Mitroulis I, Apostolidou E, Arelaki S, Tsironidou V, Giatromanolaki A, Skendros P, Konstantinides S, Ritis K. Expression of functional tissue factor by neutrophil extracellular traps in culprit artery of acute myocardial infarction. *Eur Heart J*. 2015;36:1405–1414. doi: 10.1093/eurheartj/ehv007.
 46. Li K, Ching D, Luk FS, Raffai RL. Apolipoprotein E enhances microRNA-146a in monocytes and macrophages to suppress nuclear factor- κ B-driven inflammation and atherosclerosis. *Circ Res*. 2015;117:e1–e11. doi: 10.1161/CIRCRESAHA.117.305844.
 47. Cheng HS, Besla R, Li A, et al. Paradoxical suppression of atherosclerosis in the absence of microRNA-146a. *Circ Res*. 2017;121:354–367. doi: 10.1161/CIRCRESAHA.116.310529.

Highlights

- Neutrophil elastase plasma levels are prognostic markers of adverse cardiovascular events in anticoagulated patients with atrial fibrillation. Despite not observing a higher predictive ability of the CHA₂DS₂-VASC (cardiac failure or dysfunction, hypertension, age $\geq$ 75 [doubled], diabetes, stroke [doubled] – vascular disease, age 65–74 y and sex category [female])+NE versus the CHA₂DS₂-VASC score, the C index of the modified CHA₂DS₂-VASC score for adverse cardiovascular events was improved.
- Functional rs2431697 that induces a decreased level of miR-146a promotes neutrophil extracellular trap formation in human neutrophils in vitro, suggesting for the first time the implication of a miRNA in NETosis.

Arteriosclerosis, Thrombosis, and Vascular Biology



JOURNAL OF THE AMERICAN HEART ASSOCIATION

MiR-146a Regulates Neutrophil Extracellular Trap Formation That Predicts Adverse Cardiovascular Events in Patients With Atrial Fibrillation

Ana B. Arroyo, Ascensión M. de los Reyes-García, José M. Rivera-Caravaca, Patricia Valledor, Nuria García-Barberá, Vanessa Roldán, Vicente Vicente, Constantino Martínez and Rocío González-Conejero

Arterioscler Thromb Vasc Biol. 2018;38:892-902; originally published online February 8, 2018;
doi: 10.1161/ATVBAHA.117.310597

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2018 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

<http://atvb.ahajournals.org/content/38/4/892>

Data Supplement (unedited) at:

<http://atvb.ahajournals.org/content/suppl/2018/02/06/ATVBAHA.117.310597.DC1>

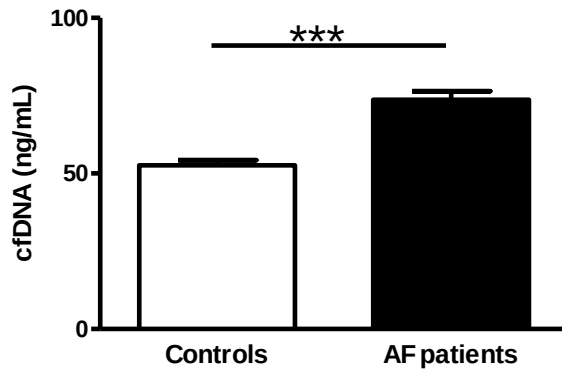
Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Arteriosclerosis, Thrombosis, and Vascular Biology* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the [Permissions and Rights Question and Answer](#) document.

Reprints: Information about reprints can be found online at:
<http://www.lww.com/reprints>

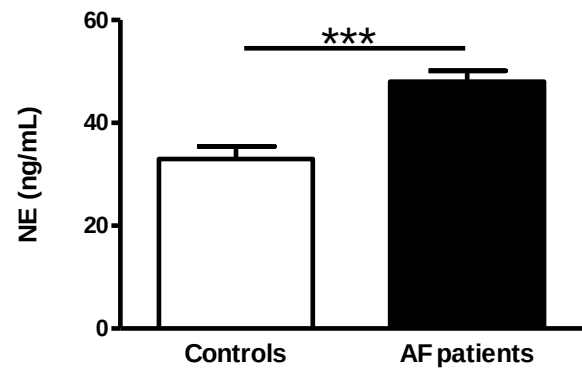
Subscriptions: Information about subscribing to *Arteriosclerosis, Thrombosis, and Vascular Biology* is online at:
<http://atvb.ahajournals.org/subscriptions/>

Supplemental data

A



B



C

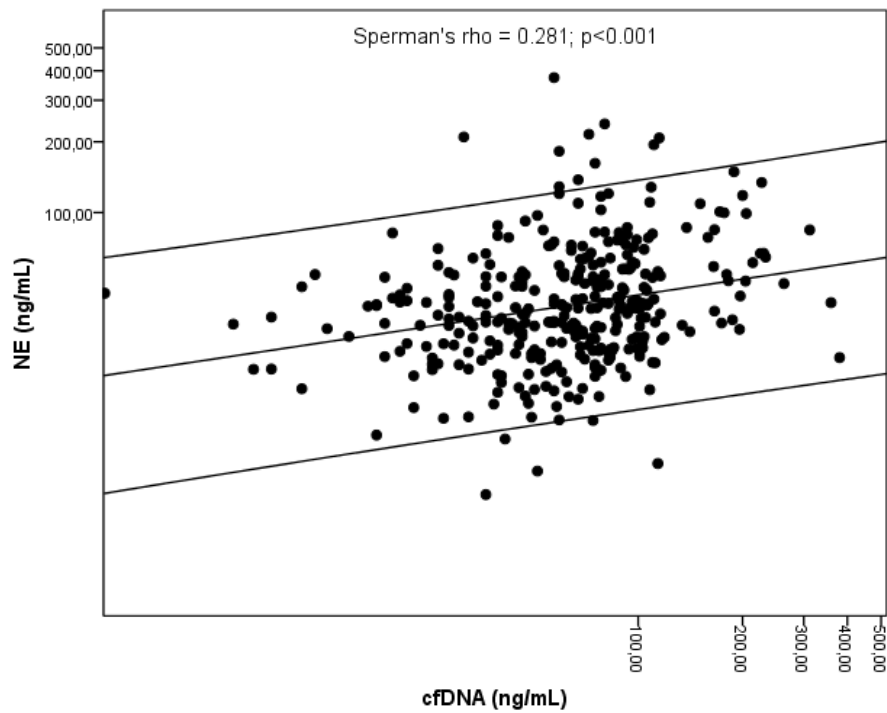


Figure I. Levels of plasma NETs markers in AF patients and controls. (A) CfDNA, (B) NE. Controls n=100, AF patients n=336. Data represent mean $\pm$ SEM. (C) Non parametric correlation between NE and cfDNA in AF patients. (*) $p < 0.001$.**

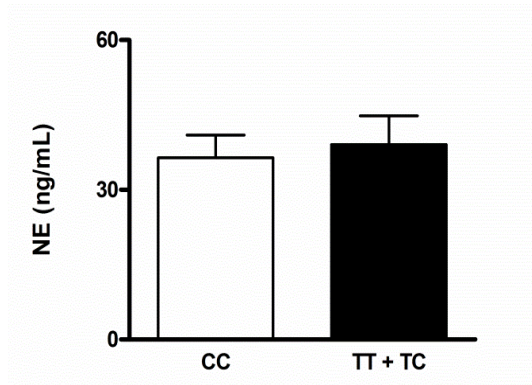
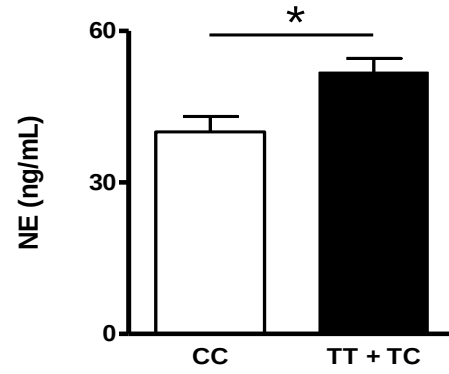
A**B**

Figure II. Plasma NE levels associated to rs2431697 genotype. (A) Healthy controls: CC (n=16), TT+TC (n=22). **(B)** AF patients: CC (n=63), TT+TC (n=252). Data represent mean $\pm$ SEM; (*) $p < 0.05$.

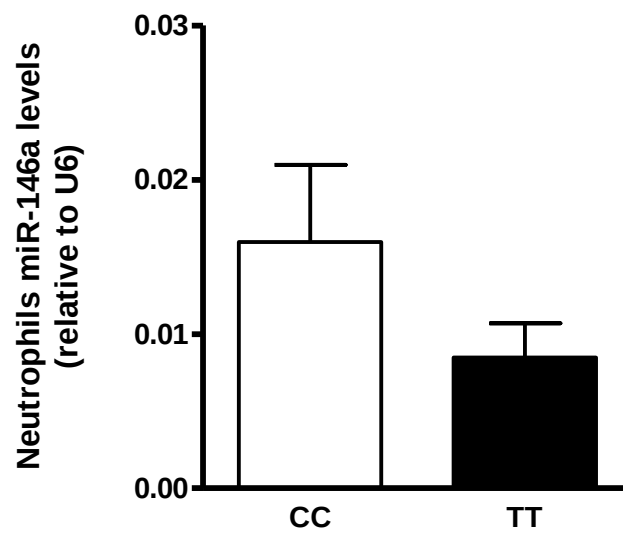


Figure III. Neutrophils miR-146a levels in healthy subjects according to rs2431697 genotype. MiR-146a expression levels from neutrophils were measured by qRT-PCR (healthy volunteers n=7/per genotype). The $2^{-\Delta Ct}$ method was followed to calculate the relative abundance of miR-146a compared with endogenous control *U6*. Data represent mean $\pm$ SEM.

Supplemental Table I. Integrated discrimination improvement and net reclassification improvement of the modified CHA₂DS₂-VASc score (by addition of neutrophil elastase) compared with the original CHA₂DS₂-VASc.

	IDI	95% CI	<i>p</i>	NRI	95% CI	<i>p</i>
All-cause mortality						
CHA ₂ DS ₂ -VASc + NE	0.024	0.004 / 0.045	<0.001	0.252	0.037 / 0.378	0.020
Cardiovascular mortality						
CHA ₂ DS ₂ -VASc + NE	0.020	-0.005 / 0.063	0.259	0.402	-0.274 / 0.721	0.328
Composite of cardiovascular events						
CHA ₂ DS ₂ -VASc + NE	0.016	-0.003 / 0.040	0.149	0.230	-0.082 / 0.365	0.139
Ischaemic stroke						
CHA ₂ DS ₂ -VASc + NE	0.016	-0.066 / 0.046	0.179	0.258	-0.135 / 0.450	0.159

CI: confidence interval; IDI: integrated discrimination improvement; NRI: net reclassification improvement; NE: neutrophil elastase

