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FACULTY OF HEALTH SCIENCES

SCHOOL OF MEDICINE

Department of Medicine & Research Laboratory of Internal Medicine

Doctoral Thesis

«Prediction of atrial fibrillation in patients with Embolic Stroke of Undetermined Source»

by

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10. **External Performance of the HAVOC Score for the Prediction of New Incident Atrial Fibrillation,** Ntaios G, Perlepe K, Lambrou D, Sirimarco G, Strambo D, Eskandari A, Karagkiozi E, Vemmou A, Koroboki E, Manios E, Makaritsis K, Vemmos K, Michel P. Stroke. 2020 Feb;51(2):457-461.

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13. **Renal Function and Risk Stratification of Patients With Embolic Stroke of Undetermined Source,** Ntaios G, Lip GYH, Lambrou D, Michel P, Perlepe K, Eskandari A, Nannoni S, Sirimarco G, Strambo D, Vemmos K, Koroboki E, Manios E, Vemmou A, Rodríguez-Campello A, Cuadrado-Godia E, Roquer J, Arnao V, Caso V, Paciaroni M, Diez-Tejedor E, Fuentes B, Rodríguez Pardo J, Arauz A, Ameriso SF, Pertierra L, Gómez-Schneider M, Hawkes MA, Bandini F, Chavarria Cano B, Mohedano AMI, García Pastor A, Gil-Núñez A, Putaala J, Tatlisumak T, Barboza MA, Karagkiozi E, Makaritsis K, Papavasileiou V, Stroke. 2018

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«Prediction of atrial fibrillation in patients with Embolic Stroke of Undetermined Source»

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Abstract

Embolic Strokes of Undetermined Source (ESUS) constitute a new clinical entity with emerging scientific interest mainly because of their high incidence and their high rate of stroke recurrence. Among potential embolic sources in ESUS, attention has focused on covert atrial fibrillation (AF) due to the significant frequency of AF detection during follow-up, which seems to be analogous to the duration of cardiac monitoring. Based on the above, it has been hypothesized that ESUS are just hidden AF strokes because of inadequate cardiac monitoring, but this hypothesis has been recently tested in 2 large, randomized trials who assessed the superiority of anticoagulant over antiplatelet treatment in this population and the result was negative. Consequently, cardiac monitoring beyond 24 hours continues to be recommended by guidelines but the criteria for the selection of patients for more prolonged monitoring remain debatable, especially given the high cost and the low availability of some of the detection methods. In this context, the present research aimed to develop a tool that can help the identification of ESUS patients who have high or low probability of new incident AF and hence support a strategy of more personalized allocation of the available resources. To accomplish this, a large range of information about consecutive, well-defined, ESUS patients from 3 stroke registries (in Athens, Larissa, and Lausanne) has been gathered and, having as main outcome new incident AF, a multivariate stepwise regression analysis with forward selection of covariates has been performed. The result was the derivation of a multivariate model and subsequently of a score, the AF-ESUS score, that comprises of eight readily available covariates, including clinical, echocardiographic, electrocardiographic, brain and vascular

imaging parameters, and has high sensitivity and high negative predictive value to identify a large proportion of ESUS patients in the cohort of the study, who have low probability of new incident AF. Having been validated internally as well as externally in an independent cohort of ESUS patients with prolonged cardiac monitoring, the AF-ESUS score could potentially assist clinical decisions about the selection of patients for the use of available cardiac rhythm monitoring resources or guide the design of future trials aiming to find the appropriate antithrombotic treatment in subgroups of ESUS population. Apart from the creation of a prognostic model for AF detection, in the course of analyzing the plethora of data collected, several other interesting findings have arisen from the present research, concerning ESUS, their characteristics, their potential embolic sources as well as their risk for stroke recurrence.

Περίληψη

Τα εμβολικά εγκεφαλικά επεισόδια απροσδιόριστης προέλευσης (ESUS) αποτελούν μια νέα κλινική οντότητα που απασχολεί την επιστημονική κοινότητα, κυρίως εξαιτίας της υψηλής επίπτωσής τους και του υψηλού ποσοστού υποτροπής εγκεφαλικού επεισοδίου. Μεταξύ των πιθανών εμβολικών πηγών στα ESUS, η προσοχή έχει επικεντρωθεί στην λανθάνουσα κολπική μαρμαρυγή (ΚΜ) λόγω της αυξημένης συχνότητας ανίχνευσής της κατά τη διάρκεια του follow-up, η οποία φαίνεται να βαίνει ανάλογα με το συνολικό χρόνο παρακολούθησης του καρδιακού ρυθμού. Με βάση τα παραπάνω, δημιουργήθηκε η υπόθεση ότι τα ESUS είναι απλώς 'συγκεκαλυμμένα' καρδιοεμβολικά εγκεφαλικά επεισόδια λόγω ανεπαρκούς καρδιακής παρακολούθησης, αλλά η υπόθεση αυτή δοκιμάστηκε πρόσφατα μέσω 2 μεγάλων, τυχαιοποιημένων κλινικών μελετών που αξιολόγησαν την υπεροχή της αντιπηκτικής έναντι της αντιαιμοπεταλιακής αγωγής στους ασθενείς αυτούς και το αποτέλεσμα ήταν αρνητικό. Κατά συνέπεια, ο έλεγχος του καρδιακού ρυθμού πέραν των 24 ωρών εξακολουθεί να συνιστάται από τις οδηγίες, αλλά τα κριτήρια για την επιλογή των ασθενών για παρατεταμένη παρακολούθηση παραμένουν αμφίβολα, ιδίως λόγω του υψηλού κόστους και της χαμηλής διαθεσιμότητας ορισμένων από τις μεθόδους ανίχνευσης. Σε αυτό το πλαίσιο, στόχος της παρούσας έρευνας ήταν η δημιουργία ενός εργαλείου που θα μπορούσε να βοηθήσει στην αναγνώριση ασθενών με μικρότερη η μεγαλύτερη πιθανότητα εμφάνισης κολπικής μαρμαρυγής επιτρέποντας έτσι μια στρατηγική πιο εξατομικευμένης κατανομής των διαθέσιμων πόρων. Για να επιτευχθεί αυτό, συλλέξαμε ένα μεγάλο εύρος πληροφοριών από διαδοχικούς ασθενείς με ESUS σε 3 βάσεις καταγραφής εγκεφαλικών επεισοδίων

(Αθήνα, Λάρισα και Λωζάνη) και έχοντας ως κύριο αποτέλεσμα την εμφάνιση κολπικής μαρμαρυγής, πραγματοποιήσαμε πολυμεταβλητή ανάλυση παλινδρόμησης. Το αποτέλεσμα ήταν η δημιουργία ενός πολυπαραγοντικού μοντέλου και εν συνεχεία ενός σκορ, αποτελούμενου από 8, εύκολα υπολογίσιμους συντελεστές, που συνδυάζουν κλινικά, ηχοκαρδιογραφικά, ηλεκτροκαρδιογραφικά και απεικονιστικά δεδομένα. Το προτεινόμενο σκορ έχει υψηλή ευαισθησία και υψηλή αρνητική προγνωστική αξία για τον εντοπισμό ενός μεγάλου ποσοστού ασθενών με ESUS στη μελέτη μας που έχουν χαμηλή πιθανότητα εμφάνισης ΚΜ σύμφωνα με την προγνωστική ικανότητα του μοντέλου. Έχοντας επικυρωθεί εσωτερικά αλλά και εξωτερικά σε ένα ανεξάρτητο πληθυσμό ESUS ασθενών υπό μακροχρόνια καρδιακή παρακολούθηση, το προτεινόμενο προγνωστικό σκορ, θα μπορούσε ενδεχομένως να βοηθήσει στην λήψη κλινικών αποφάσεων σχετικά με την επιλογή ασθενών για παρατεταμένη παρακολούθηση καρδιακού ρυθμού, ή και να βοηθήσει στο σχεδιασμό μελετών με στόχο την ανάδειξη της βέλτιστης αντιθρομβωτικής αγωγής σε υποπληθυσμούς ESUS ασθενών. Εκτός από τη δημιουργία ενός προγνωστικού μοντέλου για την ανίχνευση ΚΜ, στα πλαίσια ανάλυσης της πληθώρας των δεδομένων που συλλέχθηκαν, από την παρούσα έρευνα προέκυψαν διάφορα άλλα ενδιαφέροντα ευρήματα σχετικά με τα χαρακτηριστικά, τις πιθανές εμβολικές πηγές καθώς και τον κίνδυνο για υποτροπή του εγκεφαλικού επεισοδίου των ασθενών με ESUS,.

Table of contents

1 List of figures

2 List of tables

3 List of abbreviations

AC	*Atrial Cardiopathy*
ACA	Anterior Cerebral Artery
AF	Atrial Fibrillation
AHA/ASA	American Heart Association/American Stroke Association
AHRE	Atrial High-Rate Episodes
ASA	Atrial Septal Aneurysm
CI	Confidence Intervals
CIEDs	Cardiac Implantable Electronic Devices
CRP	C- Reactive Protein
CT	Computer Tomography
DOAC	Direct Oral Anticoagulant
ECG	Electrocardiogram
ESUS	Embolic Stroke of Undetermined Source
HR	Hazard Ratio
ICD	Implantable Cardioverter Defibrillator

ICH	Intracerebral Hemorrhage
ILR	Implantable Loop Recorder
INR	International Normalized Ratio
IQR	Interquartile Range
LV	Left Ventricle
LVEF	Left Ventricular Ejection Fraction
MCA	Middle Cerebral Artery
MI	Myocardial Infraction
MRI	Magnetic Resonance Imaging.
NIHSS	National Institutes of Health Stroke Scale
NOAC	Novel Oral Anticoagulant
NPV	Negative Predictive Value
NT-ProBNP	N-Terminal Pro hormone B-type natriuretic peptide
OR	Odds Ratio
PAF	Paroxysmal Atrial Fibrillation
PCA	Posterior Cerebral Artery
PES	Potential Embolic Source

PFO	Patent Foramen Ovale
PTFV1	P wave Terminal Force in lead V1
SVE	Supraventricular Extrasystole
TIA	Transient Ischemic Attack
VIF	Variance Inflation Factor
VKA	Vitamin K Antagonist

4 Introduction

A new clinical entity termed *Embolic Stroke of Undetermined Source* (ESUS) was recently introduced by the Cryptogenic Stroke/ESUS International Working Group, which describes stroke patients for whom the source of embolism remains undetected despite thorough investigation; potential embolic sources include covert atrial fibrillation, diseases of the mitral and aortic valves, the left cardiac chambers, the proximal cerebral arteries of the aortic arch and the venous system via paradoxical embolism (1). ESUS has been initially proposed as a potential therapeutic entity with a possible indication for anticoagulation, a hypothesis which has been currently tested in two randomized controlled trials(2)(3).

ESUS is defined as a visualized non-lacunar brain infarct in the absence of: a) extracranial or intracranial atherosclerosis causing ≥50% luminal stenosis in arteries supplying the area of ischemia, b) major-risk cardioembolic source, and c) any other specific cause of stroke (e.g. arteritis, dissection, migraine/vasospasm, drug misuse)(1) . Major risk sources of cardioembolism include permanent or paroxysmal atrial fibrillation, sustained atrial flutter, intracardiac thrombus, prosthetic cardiac valve, atrial myxoma or other cardiac tumors, mitral stenosis, recent (<4 weeks) myocardial infarction, left ventricular ejection fraction less than 30%, valvular vegetations, or infective endocarditis(1).

The reported frequency of ESUS ranges from 9% to 25% of ischemic strokes, averaging 17%(4). Recently, a descriptive analysis of an ESUS population derived from the Athens Stroke Registry reported that among the overall ischemic stroke population,

10% of patients were classified as ESUS (5). These strokes were of mild-moderate severity and covert AF was identified as the underlying etiopathogenetic mechanism in approximately 40% of ESUS patients. The mortality risk in ESUS patients is lower compared to patients with cardioembolic stroke despite similar rates of stroke recurrence(6).

Also, the risk of stroke recurrence is higher in ESUS patients than in patients with non-cardioembolic strokes which could be a sign that the current antithrombotic strategy of treating ESUS patients with antiplatelets is suboptimal. Indeed, currently, it is not clear whether antiplatelets or anticoagulants are the ideal antithrombotic strategy in all ESUS patients. Recently, two international, phase III, double-blind, randomized, controlled clinical trial were launched aiming to investigate whether anticoagulant treatment is superior to antiplatelet treatment for the secondary prevention in ESUS patients: the RE-SPECT ESUS (Randomized Evaluation in Secondary stroke Prevention Comparing the Thrombin inhibitor dabigatran etexilate versus aspirin in Embolic Stroke of Undetermined Source) trial (3) and the NAVIGATE ESUS (New Approach riVaroxaban Inhibition of factor Xa in a Global trial vs. Asa to prevent Embolism in Embolic Stroke of Undetermined Source) trial comparing rivaroxaban versus aspirin in ESUS patients (2). If any of these trials had been positive, it would have signalled a paradigm shift towards oral anticoagulation for secondary stroke prevention in ESUS patients. However, none of these two trials showed superiority of anticoagulation, which means that the detection of covert AF remains the main indication for anticoagulation in this stroke population, and therefore, cardiac rhythm monitoring beyond 24 hours continues to be recommended(7), given that the detection of atrial fibrillation (AF) increases with increasing duration of cardiac rhythm monitoring in stroke survivors (8–10). Still, surveys showed that only a small proportion

of ESUS patients receive prolonged cardiac monitoring, mainly due to limited availability of technical and human resources, as well as due to the considerable cost for some of the available monitoring (11,12).

In this context, the identification of ESUS patients who have high or low probability of new diagnosis of AF could potentially guide the selection of patients for more intense and prolonged monitoring and hence, increase the diagnosis of covert AF, influence the right choice of antithrombotic treatment, and in this way contribute to the reduction of stroke recurrence rate in ESUS.

The present research constitutes an attempt to accomplish this identification through a multicenter retrospective observational study of a large number of ESUS patients. Chapter 5 analyzes the theoretical framework that support the conceptualization of the research study. Chapter 6 explains in detail the methodology followed for data collection, patients' follow-up and statistical analysis, while chapter 7 presents the results coming out from the analysis. Finally, chapter 8 discusses the meaning, the relevance and the practical implications of the results, deriving from the present research.

5 Literature review/theoretical framework

Ischemic stroke has long been recognized to result from several different causes of obstruction of the arteries supplying the brain. The classic TOAST stroke subtype classification system denotes five sources of ischemic stroke: large-artery atherosclerosis (embolus/thrombosis), cardioembolism, Small-vessel occlusion (lacune), stroke of other unusual etiology (eg. dissection, arteritis) and stroke of unknown cause (13).

Strokes of unknown cause, usually termed cryptogenic, constitute an important proportion of about 25% of all ischemic strokes. This proportion varies according to the patient population (largest in young stroke and transient ischemic attack cohorts), the criteria for classification as cryptogenic, and the extent of diagnostic assessment (14–33). Despite the reported, in different studies, high incidence of cryptogenic strokes, the term cryptogenic is not precise. It constitutes not a defined construct with a generally accepted definition or a specified diagnostic assessment, rather than a vague entity that, based on TOAST classification, includes a) strokes of unknown cause because of incomplete evaluation, b) strokes with 2 or more identified causes, and c) really cryptogenic strokes. i.e. strokes of unknown cause despite thorough diagnostic workup (13).

This ambiguous definition is perhaps the reason that, despite the high incidence of cryptogenic strokes and their importance (about 300 000 incident cases annually in North America and Europe), there has been little progress in secondary prevention in this stroke population during the past two decades. No randomized trials devoted specifically to cryptogenic stroke have been designed to define optimum antithrombotic prophylaxis. In this context, in 2014 the Cryptogenic Stroke/ESUS International Working Group

introduced the term Embolic Stroke of Undetermined Source (ESUS) so as to give a precise definition for really cryptogenic strokes, useful in clinical practice as well as in research, and especially useful for the design of randomized trials aiming to clarify the proper therapeutical approach in this stroke population (1).

According to the proposed definition, ESUS is a non-lacunar brain infarct detected by CT or MRI, in the absence of (a) extracranial/intracranial atherosclerosis causing ≥50% luminal stenosis in the arteries which supply the ischemic territory; (b) major-risk cardioembolic source (namely permanent or paroxysmal AF, sustained atrial flutter, intracardiac thrombus, prosthetic cardiac valve, atrial myxoma or other cardiac tumors, mitral stenosis, recent (<4 weeks) MI, LVEF <30%, valvular vegetations or infective endocarditis), and (c) any other specific cause of stroke (eg. arteritis, dissection, migraine/vasospasm, drug misuse) (1).

As it derives from the definition above, investigations to establish a diagnosis of ESUS must be sufficient to exclude major risk cardioembolic sources, proximal occlusive atherosclerosis, and lacunar strokes due to cerebral small artery disease. The proposed diagnostic approach includes: a) visualisation of the brain infarct by neuroimaging (CT or MRI) to confirm the diagnosis of ischemic stroke (stroke mimics are not rare) and to exclude lacunar infarcts based on infarct appearance and topography, b)12-lead ECG and cardiac monitoring for ≥24 hours with automated rhythm detection to exclude atrial fibrillation, c)precordial echocardiography to exclude intraventricular thrombus or other major-risk cardioembolic source and d)imaging of both the extracranial and intracranial arteries supplying the area of brain ischemia (catheter, MR, or CT angiography, or cervical duplex plus transcranial doppler ultrasonography) to exclude occlusive atherosclerosis.

This diagnostic assessment is simple, is already routinely done at many stroke centers and could be applied widely if effective interventions for secondary stroke prevention in patients with ESUS were established (1).

The two main characteristics of ESUS, that makes them an important stroke subgroup and support the rationale for ongoing and future research in this population, are their high incidence and their considerable rate of stroke recurrence (34). The reported frequency of ESUS, in different studies, ranges from 9% to 25%, averaging 17% of all ischemic strokes, while the annualized recurrent stroke rate rises up to 4 -5% (4,5). The probability of stroke recurrence in ESUS seems to be similar to cardioembolic strokes but significantly higher compared with all other types of noncardioembolic stroke (6). In addition, patients with ESUS are relatively younger, compared with other ischemic stroke subtypes (mean age 65 years), and have, on average, minor strokes, consistent with small emboli (mean NIH stroke score at stroke onset: 5) (4).

ESUS encompasses diverse emboligenic mechanisms and pathogeneses. The main pathologies that could be etiologically associated with ESUS are presented in **table 1**, and could be broadly categorized in 5 embolic sources: covert AF, minor risk cardioembolic sources, arteriogenic emboli, paradoxical embolism, and cancer (1). The presence of a potential embolic source in an ESUS patient should not be automatically presumed as its actual cause, given that it may be simply an innocent bystander (35).

Table 1: *Causes of embolic strokes of undetermined source*

Minor-risk potential cardioembolic sources Mitral valve • Myxomatous valvulopathy with prolapse • Mitral annular calcification Aortic valve • Aortic valve stenosis • Calcific aortic valve Non-atrial fibrillation atrial dysrhythmias and stasis • Atrial asystole and sick-sinus syndrome • Atrial high-rate episodes • Atrial appendage stasis with reduced flow velocities or spontaneous echodensities Atrial structural abnormalities • Atrial septal aneurysm • Chiari network Left ventricle • Moderate systolic or diastolic dysfunction (global or regional) • Ventricular non-compaction • Endomyocardial fibrosis
Covert paroxysmal atrial fibrillation
Cancer-associated • Covert non-bacterial thrombotic endocarditis • Tumor emboli from occult cancer
Arteriogenic emboli • Aortic arch atherosclerotic plaques • Cerebral artery non-stenotic plaques with ulceration
Paradoxical embolism • Patent foramen ovale • Atrial septal defect • Pulmonary arteriovenous fistula

Minor-risk cardioembolic sources include moderate left ventricular dysfunction (36), left ventricular non-compaction (37), cardiac valvular diseases including myxomatous mitral valve disease with prolapse (38), mitral annular calcification (39–41) and calcific aortic valve with or without stenosis (42), atrial septal aneurysms (43–46), atrial dysrhythmias including atrial asystole, sick-sinus syndrome and atrial high-rate episodes not fulfilling AF criteria (47,48) and atrial/ atrial appendage stasis with reduced flow velocities or spontaneous echodensities (49).

Left ventricular dysfunction as an underlying cause of ESUS can be either diastolic, with preserved ejection fraction (50), or systolic with global or regional wall motion abnormalities or aneurysms as after myocardial infarction (51). Diseased contractility, atrial or/and ventricular dilatation and endothelial dysfunction, all of which can promote atrial stasis and thrombus formation, are believed to comprise the pathophysiological connection between left ventricular disease and ESUS (52). Based on a recent meta-analysis of randomized controlled trials estimating the effect of oral anticoagulation in stroke risk reduction in patients with heart failure and sinus rhythm, anticoagulation-treated patients had lower stroke risk compared with patients assigned to antiplatelets or placebo, something that is in accordance with the believed causal association between left ventricular disease and ESUS (53). However, the significant increase in major bleeding risk reported in anticoagulated patients, counterpoises any beneficial effect of oral anticoagulation and renders anticoagulants not a recommended treatment in these stroke population for the time being (53).

Atrial "dysfunction" as a risk factor for embolic events has been well investigated over the last few years and there is emerging evidence indicating that thrombi formation in the diseased left atrium, can occur even in the absence of atrial fibrillation (50). The hypothesis that anatomic and physiological derangements of the left atrium, like atrial enlargement, endothelial dysfunction and myocardial fibrosis, constitute a sufficient condition for thrombus formation is gaining ground and is supported by the fact that spontaneous echocardiographic contrast, considered an echocardiographic marker of a prothrombotic tendency, and thrombus formation can be present in these conditions prior to the occurrence of frank atrial fibrillation (49,50,54). Several markers have been used for the definition of atrial cardiopathy including biomarkers (such as NT-proBNP) (55),

indexes from cardiac magnetic resonance imaging (56), electrocardiographic measures (such as P wave Terminal Force in lead V1 ($PTFV_1$)) and echocardiographic measurements (such as left atrial size) (57–59). Despite the strong suggestion that atrial cardiopathy can exist and cause thromboembolism even in the absence of AF, the effectiveness of anticoagulant therapy in this stroke population has not yet been demonstrated. However recently, a new randomized, double-blind, active-control, phase 3 trial, the AtRial Cardiopathy and Antithrombotic Drugs In Prevention After Cryptogenic Stroke (ARCADIA) trial, has been launched, aiming to test if the direct-acting oral anticoagulant apixaban is superior to aspirin for the prevention of recurrent stroke in patients who have evidence of atrial cardiopathy and a recent stroke of unknown cause (60).

It is worth noting that, in about half of patients with ischemic stroke or transient ischemic attack, at least one of the above-mentioned minor-risk cardiac abnormalities can be recognized (61–64). Despite their frequency, these common cardiac lesions are associated with a low rate of initial stroke, and are more often incidental than the actual cause of stroke (65). Even if, each of these abnormalities has a recognized association with emboli formation in the general population, and can therefore be a source of ESUS, at the individual patient level, the presence of these lesions cannot be reliably connected with the index stroke. Consequently, for the time being, antithrombotic therapy is not recommended for primary stroke prevention for patients in whom minor-risk sources have been identified (1).

As concerns arteriogenic embolism, it has been shown that, non-stenotic carotid plaques (i.e. plaques causing <50% diameter stenosis) are present in a high proportion of all ischemic stroke patients (66) but carry a low absolute risk of stroke (67). However,

bibliography supports that non-stenotic carotid, as well as vertebral artery plaques, can be a source of embolism, especially if ulcerated and irregular (68–70). This is reinforced by several studies and meta-analysis highlighting the higher frequency of detection of nonstenosing complicated plaques or plaques with “high risk features” in the carotid arteries ipsilateral to the stroke rather than contralateral (68,71). In accordance with the above, in the NAVIGATE ESUS trial carotid plaque was much more often present ipsilateral to the qualifying ischemic stroke than contralateral (72). Expect from non-stenotic carotid plaques, aortic arch atheroma (present in about 30% of the stroke-prone age group) has also been correlated with cardioembolic stroke in different studies (73,74). Actually, there is emerging evidence showing that the role of supracardiac atherosclerosis (definition that includes atherosclerotic plaque in the carotid, vertebrobasilar, and intracranial arteries, or the aortic arch) is larger than it was initially perceived (75). Recently, an analysis of consecutive emboli retrieved during mechanical thrombectomy showed that large artery atherosclerosis and cryptogenic patients had a similar proportion of platelet-rich clots, which was significantly higher compared with cardioembolic cases, supporting the argument that supracardiac atherosclerosis is the cause of stroke in a significant proportion of ESUS (76). Despite the fact that evidence about the role of supracardiac atherosclerosis in patients with ESUS is accumulating, there are still many questions which remain unanswered, and imaging of these lesions via CT or MRI angiography, is not routinely done in most patients because there are no evidence-based management implications. An important point is the assessment of the value of plaque characteristics in stroke risk stratification and subsequently, in clinical decision-making (75). Several ongoing prospective studies are expected to provide more evidence on this topic (77,78).

Paradoxical embolism is also considered to be a potential cause of ESUS. The foramen ovale is an obligatory channel during fetal life that allows placental oxygenated blood to reach the arterial circulation of the fetus. When there is incomplete postnatal fusion of the septum primum and secundum, a PFO is formed. The presence of a patent foramen ovale (PFO) with either transient or continuous right-to-left shunt can potentially lead to paradoxical embolism. The PFO serves as a potential conduit for venous emboli to cross into the left atrium and eventually to the arterial circulation. PFO is the most common congenital cardiac abnormality present in approximately 25% of the population (79) and responsible for up to 95% of right-to-left shunts (80). The causal relationship between PFO and cryptogenic stroke has historically been controversial. The condition by itself has not been shown to increase the risk of ischemic stroke (81). Yet, results of case control studies have consistently shown a higher frequency of patent foramen ovale in patients with cryptogenic stroke than in age-matched controls without stroke (43). Additionally, PFO is found in up to 40% of ischemic strokes without an identifiable cause (82,83). The above evidence suggests that paradoxical embolism through a PFO may be implicated in a considerable proportion of cryptogenic strokes.

The presence of an atrial septal aneurysm (ASA) has also been associated with cryptogenic stroke (83). An ASA is described as redundant bulging atrial septal tissue that can be caused by sustained interatrial pressure difference or can be a primary malformation involving either the fossa ovalis or the entire atrial septum. It is objectively defined on echocardiography as 15-mm of total septal tissue excursion or a 10-mm protrusion into either atrium from the septal midline (84). Several studies have linked the presence of ASA to stroke while the combination of a PFO and ASA has been shown to be a significant risk factor for recurrent stroke (83).

Despite the above data, the early randomized trials of percutaneous PFO closure in cryptogenic stroke patients [CLOSURE I (Evaluation of the STARFlex Septal Closure System in Patients with a Stroke and/or Transient Ischemic Attack due to Presumed Paradoxical Embolism through a Patent Foramen Ovale), PC (Percutaneous Closure of Patent Foramen Ovale in Cryptogenic Embolism) and REDUCE (Randomized Evaluation of Recurrent Stroke Comparing PFO Closure to Established Current Standard of Care Treatment) trials], failed to show benefit, rendering the etiological role of PFO in ESUS debatable (85–87). However, the recent randomized trials of percutaneous PFO closure (RESPECT long term follow up, CLOSE, REDUSE and DEFENCE-PFO) yielded impressive results as concerns reduction in stroke recurrence, in patients with ESUS who are <60 years, and verified the possible association between PFO and ESUS in this patient group (88–91). However, even in this age group, the presence of a PFO discovered in a cryptogenic stroke patient, does not necessarily imply causality and may simply be an incidental finding.

The need of approaching the probability that a discovered PFO is causally linked to the index stroke has led to the development of the Risk of Paradoxical Embolism (RoPE) score (92). The RoPE score is a 10-point score **(Table 2)** that stratifies patients according to the probability of having a PFO (higher the RoPE score value, higher the frequency of PFO): from the frequency of PFO observed for each value of RoPE score using the Bayes theorem it is possible to derive the PFO-attributable fraction, that is, the probability that the index event was related to the PFO, as described in the RoPE score derivation study (92). Accordingly, the higher the prevalence of PFO in a given subgroup of patients with stroke with specific features (ie, a given value of RoPE score), the higher the PFO-attributable fraction in patients with those characteristics. Despite promising data, this tool has only

been externally validated in a small population of cryptogenic stroke patients under 50 years old and never in patients with ESUS (4).

Table 2: *RoPE Score*

Characteristic	**Point**
No history of hypertension	1
No history of diabetes	1
No history of stroke or TIA	1
Nonsmoker	1
Cortical infarct on imaging (or cerebellar)	1
Age (years)	
18-19	5
30-39	4
40-49	3
50-59	2
60-69	1
≥ 70	0

Cancer constitutes another underlying potential cause of ESUS. Based on epidemiological data, historical or active cancer as a coexisting disease is present in about 10% of all ischemic stroke patients, a proportion which is expected to increase further in the next years, as life expectancy in patients with cancer increases (93). However, as is the case for other possible sources of embolism, presence of cancer cannot always be reliably connected to the index stroke, so the proportion of ESUS that are etiologically linked with cancer is likely less than the above-mentioned 10%. Nevertheless, patients with cancer, and especially patients with active cancer, have a well-established increased risk for stroke (94–97) risk that seems to be higher in the first 6 months after cancer diagnosis, in patients with distant metastases as well as in cancers most linked to venous thromboembolism, as lung and pancreatic cancer (96). It has also been observed that stroke, particularly ESUS,

can be the initial presentation of cancer (98). The potential pathophysiological mechanisms underlying cancer-mediated ESUS strokes, include tumor embolism, mechanical compression of vessels, nonbacterial thrombotic endocarditis, anemia, radiotherapy, antineoplastic treatment adverse effects, and, mainly, hypercoagulopathy, an entity predisposing to venous thromboembolism and arterial events (99). In addition, stroke may be caused by (prothrombotic) cancer treatments, surgical interventions, and distant radiotherapy. Despite its strong pathophysiological basis, an attempt to show superiority of antithrombotic over antiplatelet treatment in patients with stroke and cancer in two recent small randomized controlled trials has been proved unsuccessful; on the other hand, these studies were clearly underpowered (100,101). Hence, the role of anticoagulation in patients with ESUS and cancer remains unclear.

Among potential embolic sources in ESUS, attention has focused on covert atrial fibrillation (AF). AF is the most common form of cardiac arrhythmias and a, well studied, independent risk factor for stroke (102). AF-induced strokes are associated with greater morbidity and mortality and more severe neurological deficits (NIHSS >10) when compared to non-AF strokes (103). The former are largely avoidable with the use of oral anticoagulants, which provide an additional 40% reduction of stroke risk compared to monotherapy with antiplatelets (104).

In this point, it would be useful to clarify the definition of the currently used terms for AF. Clinical AF is defined as symptomatic or asymptomatic AF that is documented by surface ECG (105). The minimum duration of an ECG tracing of AF, required to establish the diagnosis of clinical AF, is at least 30 seconds, or entire 12-lead ECG (105). Subclinical AF refers to individuals without symptoms attributable to AF, in whom clinical AF is not

previously detected (that is, there is no surface ECG tracing of AF). Atrial High Rate Episodes (AHRE) are events, fulfilling programmed or specified criteria, detected by cardiac implantable electronic devices (CIEDs) with an atrial lead allowing automated continuous monitoring of atrial rhythm and tracings storage (105). CIED-recorded AHRE need to be visually inspected because some AHRE may be electrical artefacts/false positives. Subclinical AF as a definition includes AHRE confirmed to be atrial fibrillation, atrial flutter, or an atrial tachycardia, or AF episodes detected by insertable cardiac monitor or wearable monitor and confirmed by visually reviewed intracardiac electrograms or ECG-recorded rhythm. Device-programmed rate criterion for AHRE is ≥175 bpm, whereas there is no specific rate limit for subclinical AF. The criterion for AHRE duration is usually set at ≥5 min (mainly to reduce the inclusion of artefacts), whereas a wide range of subclinical AF duration cut-offs (from $10-20$ seconds to >24 hours) is reported in studies of the association of subclinical AF with thromboembolism. The reported duration refers to either the longest single episode or, more commonly, total duration of AHRE/subclinical AF during the specified monitoring period. Although not completely identical, the terms AHRE and subclinical AF are often used interchangeably (105).

On addition, based on the duration and termination of episodes, AF is further classified in four patterns: paroxysmal, persistent, long standing persistent and permanent. AF is defined as paroxysmal when it terminates spontaneously or with intervention within 7 days of onset, while AF that is continuously sustained beyond 7 days, is called persistent, including episodes terminated by cardioversion (drugs or electrical cardioversion) after ≥7 days. Long standing persistent is called a continuous AF of >12 months duration, when decided to adopt a rhythm control strategy. Finally, permanent AF is AF that is accepted by the patient and physician, and no further attempts to restore/maintain sinus rhythm will

be undertaken. Permanent AF represents a therapeutic attitude of the patient and physician rather than an inherent pathophysiological attribute of AF. Should a rhythm control strategy be adopted, the arrhythmia would be re-classified as 'long-standing persistent AF' (105). Of note, clinically determined AF patterns do not correspond well to the AF burden measured by long-term ECG monitoring (106,107).

Despite major advances in monitoring strategies, clinicians tend to miss the diagnoses of AF, especially if it is about short-lasting episodes (i.e. paroxysmal or subclinical AF), mainly due to its asymptomatic presentation. It is estimated that between 25 and 60% of atrial fibrillation cases are paroxysmal in nature (108) and approximately one-third of the patients affected are asymptomatic (109). However, "silent" AF can be as much implicated in stroke risk as symptomatic AF (110) while the thromboembolic risk of non-anticoagulated paroxysmal atrial fibrillation (PAF), even if not absolutely equivalent to that of more persistent forms, seems to be substantially higher in comparison to that of patients without high-rate events (111). Thus, based on the latest guidelines, patients with PAF are eligible to stroke prophylaxis in a manner identical to persistent and permanent forms (105).

The contribution of subclinical AF to the risk of stroke has been well studied and can be assessed in trials enrolling patients with implanted cardiac devices that monitor Atrial High Rate Episodes. In the Mode Selection Trial in Sinus-Node Dysfunction (MOST), patients with intracardiac therapeutic devices in whom atrial high-rate episodes (>220 bpm) lasting >5 minutes was detected, had 6.7-fold increased risk of stroke and a 2.48-fold increased risk of death (112). Based on the TRENDS (A Prospective Study of the Clinical Significance of Atrial Arrhythmias Detected by Implanted Device Diagnostics)

study, which was a prospective, observational study involving 2486 patients with ≥1 stroke risk factor receiving pacemakers or defibrillators, Atrial Tachycardia (AT)/AF burden ≥5.5 hours (defined as the longest total AT/AF duration on any given day during the prior 30-day period) appeared to double thromboembolic risk (113). The Asymptomatic Atrial Fibrillation and Stroke Evaluation in Pacemaker Patients and the Atrial Fibrillation Reduction Atrial Pacing Trial (ASSERT) enrolled 2580 patients, 65 years of age or older, with hypertension and no history of atrial fibrillation, with a recently implemented pacemaker or defibrillator, in order to prospectively evaluate whether subclinical episodes of rapid atrial rate detected by implanted devices are associated with an increased risk of ischemic stroke (114). During the first 3 months of follow-up, subclinical atrial tachyarrhythmias (defined as 190 bpm for >6 minutes), occurred in 261 patients (10.1%) which means a 2.5-fold increase in the risk for ischemic stroke and systemic embolism (114).

On the basis of the above, covert atrial fibrillation (AF) was initially conceived as perhaps the most important underlying mechanism in ESUS patients, especially given that prolonged cardiac rhythm monitoring is not a prerequisite for the definition of ESUS (34). In fact, there was a perception that ESUS strokes are potentially just hidden AF- strokes because of inadequate cardiac monitoring, leaving doubts about the accuracy of the current antithrombotic treatment in this population; nearly 90% of ESUS patients had been treated with antiplatelets (4). This belief was powered, on the one hand, by the high annualized rate of stroke recurrence in ESUS patients under antiplatelet drugs, which rises up to 4-5%/year (4), and ,on the other hand, by the fact that randomized controlled trials of prolonged cardiac monitoring like the CRYSTAL-AF (Study of Continuous Cardiac Monitoring to Assess Atrial Fibrillation After Cryptogenic Stroke) (9), EMBRACE (30-

Day Cardiac Event Monitor Belt for Recording Atrial Fibrillation After a Cerebral Ischemic Event) (10), and Find-AF$_{RANDOMISED}$ (A Prospective, Randomized, Controlled Study to Determine the Detection of Atrial Fibrillation by Prolonged and Enhanced Holter Monitoring as Compared to Usual Care in Stroke Patients) (8), as well as observational studies (4,5) and meta-analyses (115), showed that AF may be detected in up to 30% of ESUS patients during long-term follow-up.

However, whether detected AF is etiologically linked with the index stroke remains a topic of discussion, particularly if episodes of AF are short-lasting, subclinical, or detected remotely after stroke (116). Actually, there are several facts that can be opposed to the argument of a strong causal association between detected AF and ESUS:

Firstly, as derives from the Find-AF $_{RANDOMISED}$ study, the rate of AF detection through prolonged monitoring with Holter seem to have no difference between ESUS and the rest of ischemic stroke patients (8).

Secondly, according to the results of the ASSERT-II (Subclinical Atrial Fibrillation in Older Patients - II) trial, patients≥65 years with and without prior stroke, have similar rates of detection of subclinical atrial fibrillation episodes (SCAF) ≥5 min (117). However, it has to be mentioned that, a large study in Medicare beneficiaries showed that AF incidence is higher after hospitalization for ischemic stroke than after hospitalization for hemorrhagic stroke or non-stroke conditions (118).

Thirdly, as shown across registries and trials, ESUS and AF-stroke patients seem to have major phenotypical differences, with the former being younger with milder strokes (2–5). In the Athens Stroke Registry, the mean NIHSS score of patients with ESUS was 5,

which is considerably lower compared to the mean NIHSS score of 13 in patients with cardioembolic stroke, while the mean age of ESUS patients was 68 years compared to a mean age of 76 years in cardioembolic stroke patients (5).

Fourthly, as derives from subgroup analyses of studies as ASSERT (119) and TRENDS (120), which, as already mentioned, included patients with implantable cardiac monitoring devices, only the minority of patients who experienced an embolic event (stroke or systemic embolism) had a detected episode of atrial tachycardia or AF proximal to the event, whereas some of them had a detected AF episode only after the stroke event. This strengthens the argument that some of the detected AF episodes through long-term monitoring may be coincidental and not the real source of embolism.

Apart from the above, the most important argument against the hypothesis that hidden AF is the main underlying cause in all cryptogenic stroke patients, is the fact that this hypothesis has actually been tested through different randomized controlled trials (RCTs), who compared anticoagulant and antiplatelet treatment in this stroke population, and the result was negative. The rationale behind the development of these trials was the assumption that, if most cryptogenic strokes were thromboembolic, they could benefit from oral anticoagulants, that had already demonstrated their efficacy and safety in atrial fibrillation, to reduce recurrent brain ischemia (104) (121–124).

The Stroke Prevention in Reversible Ischemia Trial (SPIRIT) was the first multicenter randomized control trial to assess the efficacy of warfarin (with an INR target of 3.0–4.5), compared with antiplatelet treatment in the secondary prevention after a cerebral ischemic event of presumed noncardiac origin (125). The trial was prematurely terminated, after inclusion of 1316 patients with a mean follow-up of 14 months, because

of an excess of the primary outcome event (death from all vascular causes, nonfatal stroke, nonfatal myocardial infarction, or nonfatal major bleeding complication) in the anticoagulated group [hazard ratio (HR) = 2.3; 95% confidence interval (95% CI): 1.6–3.5] (125). The investigators reported that bleeding risk increased by a factor of 1.43 (95% CI, 0.96–2.13) for each 0.5 unit increase of the achieved INR (125).

The Warfarin-Aspirin Recurrent Stroke Study (WARSS) was the second multicenter randomized control trial to compare warfarin (at a dose adjusted to produce an INR of 1.4 to 2.8) to aspirin 325 mg in the secondary prevention of patients with noncardioembolic ischemic stroke (27). The trial included a total of 2206 patients, and the result was no difference between the two groups in the primary endpoint of recurrent ischemic stroke or death (HR = 1.13, 95% CI: 0.92–1.38)(27). The rates of major hemorrhage were low but still more frequent in the warfarin group compared to the aspirin group (2.22 versus 1.49 per 100 patient-years; Risk Ratio = 1.48, 95% CI: 0.93–2.44) (27). In a post-hoc analysis, benefit of warfarin over aspirin was limited to brainstem-sparing posterior circulation infarcts and select cryptogenic stroke subgroup (126).

Taking into account the safety concerns of SPIRIT trial and the negative efficacy results of WARSS trial, the European/Australasian Stroke Prevention in Reversible Ischemia Trial (ESPRIT) trial randomized 1089 patients with noncardioembolic stroke to receive medium intensity warfarin treatment (INR between 2 and 3) or ASA (30–325 mg) within 6 months of stroke onset, and followed them for a mean of 4.6 years (127). Again, warfarin was not more effective than aspirin as concerns the primary efficacy composite outcome (HR = 1.02, 95% CI 0.77–1.35) or the risk of recurrent ischemic events (HR = 0.73, 95% CI: 0.52–1.01). Warfarin was also associated with increased risk of major

bleeding complications (HR = 2.56, 95% CI: 1.48–4.43) (127). In brief, any theoretical benefit of recurrent IS reduction with vitamin K antagonists has been offset by the increased risk of major and intracranial bleeding with coumadin or warfarin compared with aspirin.

In view of the more favorable safety profile of the direct thrombin inhibitor Dabigatran or factor Xa inhibitors, including Rivaroxaban, Apixaban and Edoxaban (relative risk reduction of approximately 50% in any or fatal intracranial hemorrhage compared with VKA) (128), it has been postulated that the "Non-vitamin K antagonist" or Novel Oral Anticoagulants (NOACs), or Direct Oral Anticoagulants (DOACs), as they are alternatively called, may represent a promising therapeutic option in cryptogenic stroke patients with an embolic source as their underlying stroke mechanism (1). On this basis, 2 large randomized control trials have been recently designed: NAVIGATE ESUS and RESPECT ESUS (2,3). NAVIGATE and RESPECT ESUS, differ from the above-mentioned trials not only because they compared DOACs instead of VKA as an anticoagulant therapy, but also because they were the first to include cryptogenic stroke patients based on precise criteria (i.e. ESUS patients).

NAVIGATE ESUS enrolled (from December 2014 to September 2017) a total of 7213 participants (at 459 sites) with recent ischemic stroke that was presumed to be from cerebral embolism but without arterial stenosis, lacune, or an identified cardioembolic source, and randomly assigned them to receive rivaroxaban (at a daily dose of 15 mg) or aspirin (at a daily dose of 100 mg) (2). The primary efficacy outcome was the first recurrence of ischemic or hemorrhagic stroke or systemic embolism in a time-to-event analysis and the primary safety outcome was the rate of major bleeding (2). Patients had been followed for a median of 11 months when the trial was terminated early because of a

lack of benefit with regard to stroke risk and because of bleeding associated with rivaroxaban (2). The trial concluded that rivaroxaban was not superior to aspirin with regard to the prevention of recurrent stroke after an initial embolic stroke of undetermined source (hazard ratio, 1.07; 95% confidence interval [CI], 0.87 to 1.33; P=0.52) and was associated with a higher risk of bleeding (hazard ratio, 2.72; 95% CI, 1.68 to 4.39; P<0.001) (2).

RE-SPECT ESUS enrolled, during the period from December 2014 through January 2018, at 564 sites, a total of 5390 patients who had an embolic stroke of undetermined source, and randomly assigned them to receive dabigatran (at a dose of 150 mg or 110 mg twice daily) or aspirin (at a dose of 100 mg once daily) (3). The primary outcome was recurrent stroke while the primary safety outcome was major bleeding, and the median follow-up was 19 months (3). Once again, the conclusion was that dabigatran was not superior to aspirin in preventing recurrent stroke in patients with a recent history of ESUS (hazard ratio, 0.85; 95% confidence interval [CI], 0.69 to 1.03; P=0.10) (3). In contrast with Navigate ESUS, in this trial the incidence of major bleeding was not greater in the dabigatran group than in the aspirin group (hazard ratio, 1.19; 95% CI, 0.85 to 1.66), but there were more clinically relevant nonmajor bleeding events in the dabigatran group (3).

Since RESPECT-ESUS and NAVIGATE-ESUS trials failed in demonstrating superiority of DOAC over antiplatelets in ESUS patients, the detection of AF during follow-up continues to constitute the only clear indication for anticoagulation in this stroke population and as a result, an extended search for AF in ESUS patients continues to be essential, to maximize secondary prevention. However, yet, sustainable recommendations on both the modality and length of search for AF, as well as the selection of patients for screening, are missing (129).

In the 2014 AHA/ASA Guidelines for the Prevention of Stroke in Patients With Stroke and TIA, there is weak evidence, for patients who have experienced an acute ischemic stroke or TIA with no other apparent cause, suggesting prolonged rhythm monitoring for AF for around 30 days within 6 months of the index event (Class of recommendation IIa; Level of Evidence C) (130). More recently, the 2019 AHA/ASA Guidelines for the Early Management of Patients With Acute Ischemic Stroke recommend cardiac monitoring for at least the first 24 hours after stroke onset (Class of recommendation I, level of Evidence B) but states that the effectiveness of prolonged cardiac monitoring during hospitalization after acute ischemic stroke, to guide treatment selection for prevention of recurrent stroke, is uncertain (Class of recommendation IIb, level of Evidence C) (131). On the other hand, in the latest 2019 AHA/ACC/HRS Focused Update of the 2014 AHA/ACC/HRS Guideline for the Management of Patients With Atrial Fibrillation, it is mentioned that, in patients with cryptogenic stroke (i.e., stroke of unknown cause), in whom external ambulatory monitoring is inconclusive, implantation of a cardiac monitor (loop recorder) is reasonable to optimize detection of silent AF (Class of recommendation IIA, level of Evidence B) (132). From the other part of the universe, European Society of Cardiology, through the recent 2020 Guidelines for Management of Atrial Fibrillation, recommend monitoring for AF using a short-term ECG recording for at least the first 24 hours, followed by continuous ECG monitoring for at least 72h whenever possible (Class of recommendation I, level of Evidence B), while it is reported that in selected stroke patients without previously known AF, additional ECG monitoring using long-term non-invasive ECG monitors or insertable cardiac monitors should be considered, to detect AF (Class of recommendation IIA, level of Evidence B) (105).

Not only is the optimal duration of monitoring debatable, but also the detection methods of covert AF vary greatly between studies as well as in clinical practice. Since the discovery by Dr. Holter in the early 1960s of a method to record, store, and display cardiac electric waves (133), a variety of different cardiac devices have been developed to allow monitoring of patients for prolonged periods (weeks, months, and even years). Thanks to more sophisticated designs and improvements in technology of storage and analysis, newer devices have become more user-friendly and gives the ability for more extensive monitoring and as a result greater likelihood of AF detection (134). In general, prolonged cardiac monitoring techniques can be divided in 2 large categories: noninvasive and invasive methods.

Noninvasive methods include ambulatory continuous monitors (Holter), that record and store data from ECG leads continuously over 24 to 48h, Intermittent External Patient- or Event-Activated Recorders, that are used for prolonged time periods (4 to 6 weeks) but record (save) data only when triggered by the patient, and Prolonged ambulatory ECG (mobile cardiovascular telemetry) that automatically record arrhythmic event data from ambulatory patients for an extended period of time (up to 30 days) and transmit them to an attended monitoring station (134).

From the other hand, invasive methods include subcutaneous recording systems like Implantable Loop Recorders (ILRs) and intracardiac recording systems like pacemakers and defibrillators (134). ILRs are subcutaneously implanted wireless devices that can be triggered automatically or by patient activation and permit a vary extended period of monitoring, as the implant may be left in place up to 3 years, overcoming the problems of skin irritation and patient compliance (134). Dual chamber pacemakers and implantable cardioverter-defibrillators (ICDs) have built-in algorithms to allow the detection of

supraventricular arrhythmia episodes even if the ventricular response is regular and maintains a normal rate. Once detected, these episodes are automatically recorded for later review (135). Current batteries of intracardiac devices may last >10 years. Of course, it must be noted that, the indication for implantation of these permanent devices is life-threatening arrhythmias and not just the need for extended monitoring (136).

The different methods of monitoring have their pros and cons, different rates of sensitivity and specificity and, most importantly, different cost. It is well established that a more prolonged period of cardiac monitoring, provide greater likelihood of AF detection (137–139). Nevertheless, the fact remains that devices with better sensitivity and capability of more extended monitoring, are in general more expensive. At present, Holter monitors of any duration and external loop event recorders are relatively inexpensive, while prolonged ambulatory ECG monitors (mobile cardiovascular telemetry) require a greater degree of technical support and, thus, the current cost of these devices is approximately twice that of standard ambulatory monitoring devices. Finally, the cost of an ILR device that allow monitoring for up to several years, is approximately $4000 in US with additional charges for implantation, follow up and removal (135,140) .

In the view of the above, it is extremely difficult, if not unfeasible, for all ESUS patients to be screened for AF with the best available method and for the maximum possible time period. As a matter of fact, based on a recent survey, globally, only 17% of hospitals routinely perform extended (i.e. >24 h) automated cardiac rhythm monitoring in patients with cryptogenic stroke, a proportion that even in high-income countries do not exceed 19% (12). It is therefore essential, and a matter of cost-effectiveness, to develop a strategy of selecting patients for more extensive screening, and this strategy could be guided by the identification of individuals at higher risk of developing AF. This approach is consistent

with data suggesting that the likelihood of detecting AF is higher in selected subgroups of cryptogenic stroke patients (134). The same emerges from a subgroup analysis of the RE-SPECT ESUS trial, in which patients aged >75 years had a significant benefit of lower-dose dabigatran over aspirin, favoring the hypothesis of new-developed or covert AF in this subgroup of patients (3). Hence, determining the most significant predictors of AF to select the best candidates for monitoring is crucial.

In the general population several factors have been associated with an increased risk of AF development (141–143). Apart from increasing age, that is a well known risk factor, several other comorbidities including hypertension, diabetes mellitus, heart failure, valve disease, coronary artery disease, chronic kidney disease, obesity, alcohol consumption, obstructive sleep apnea, and physical inactivity or excessive physical activity have also been related (144–149). Not only patient comorbidities but also various other clinical parameters such as demographics (male sex, white race) (150,151), ECG markers (PR interval, P wave indices including P wave duration, $PTFV_1$, P wave axis, and other measures of P wave morphology, criteria for left ventricular hypertrophy and premature atrial complexes) (152,153) as well as echocardiographic findings (left atrial enlargement, increased left ventricular wall thickness, diastolic dysfunction) (154,155) have at times been correlated with an increased risk and have been proposed as predictors of PAF presence or AF development. In an attempt to identify individuals in community at higher risk, several predictive scores of new onset AF, including different parameters, have been developed, but none of them is widely used in clinical practice (156–162).

A number of studies have also studied the identification of AF predictors in ischemic stroke patients as well as in cryptogenic stroke patients in particular. Older age

remains the strongest predictor of AF, identified in the majority of relevant studies (108,163–169). $CHADS_2$ [Congestive heart failure, Hypertension, Age ≥75 years, Diabetes mellitus, Stroke (double weight)] and CHA_2DS_2-VASc [Congestive heart failure, Hypertension, Age ≥75 years (double weight), Diabetes mellitus, prior Stroke (double weight), Vascular Disease, Age 65-74 years, Sex category] scores, that include age, sex and several other comorbidities from patients' medical history as hypertension, diabetes mellitus, prior stroke/transient ischemic attack, congestive heart failure and vascular disease (including coronary artery and peripheral artery disease), have also been enough studied and seem to be well correlated with new onset AF after ischemic stroke (170–173). Of course, most of the individual components of the above scores, separately, have also been related to poststroke AF diagnosis. Nevertheless, some of these risk factors, such as diabetes mellitus and vascular disease, are also considered to be predisposing factors of noncardioembolic ischemic stroke (26), so may actually be associated with a lower risk of finding AF after ischemic stroke (171).

As concerns electrocardiographic parameters, a couple of studies have assessed the association of PR interval and P wave morphology (168,174,175) but excessive supraventricular ectopic activity has consistently been associated with an increased risk of AF detection (176–178). Based on a retrospective analysis of the EMBRACE trial, the number of atrial premature beats on a routine 24-hour Holter ECG is a strong dose-dependent independent predictor of prevalent subclinical AF (179) while based on the Copenhagen Holter Study, excessive supraventricular ectopic activity is also associated with clinical AF and a poor prognosis (180).

Concerning cardiac imaging, left atrial enlargement, usually measured by transthoracic echocardiography, consists the best defined predictor of AF in stroke and cryptogenic stroke patients (166,177,181,182) while the role of the atrial appendage's morphology and its correlation with thrombogenicity is under investigation (183). Compared to transthoracic echocardiography, transesophageal echocardiography allows a better evaluation of atrial structures, aortic arch atheroma and PFO presence (184), that seem to have a negative correlation with AF detection, but is not routinely performed in cryptogenic stroke patients. Valvular abnormalities, in particular rheumatic mitral valve stenosis or severe mitral and tricuspid valve insufficiency as well as spontaneous echo contrast or solid thrombi in the atrium have also been reported as predictors of AF in cryptogenic stroke (185). The combination of left atrial volume index and atrial function has been demonstrated to be predictive for detection of AF (186) whereas, the role of left atrial deformation characteristics, using tissue doppler and speckle tracking techniques, as atrial longitudinal strain, is currently being examined (187,188).

With regard to stroke characteristics, NIHSS seems to have a possible correlation (164,189). Although topography of the acute infract, on brain imaging, did not appear, in a retrospective analysis of CRYSTAL-AF trial, to be clearly associated with AF detection (190), radiographic evidence of previous brain infracts, especially if multiple and multifocal, as well as presence of leukoaraiosis, seems to be (108,163,167,190,191). It is a fact that secondary hemorrhagic transformation on brain imaging (i.e. hemorrhagic transformation of an ischemic stroke) is more common in patients with known AF, so it may suggest an embolic origin from the heart (192). However, the emergence of hemorrhagic conversion as a predisposing factor for AF detection in cryptogenic stroke is not a consistent finding, although it has been described (163).

Several studies vale also tested the predictive value of different biomarkers related with atrial dilatation, inflammation and impaired cardiac or endothelial function, as natriuretic peptides ,troponins, interleukin-6, C-reactive protein, D-dimer, glomerular filtration rate and cystatin C (185). From the above mentioned biomarkers, the acute phase reactant C-reactive protein (CRP) and the natriuretic peptides N-terminal pro B-type natriuretic peptide (Nt-proBNP) and B-type natriuretic peptide (BNP) seem to have the most important correlation (193,194), and thus, have been proposed as indicators of the need for prolonged monitoring after ischemic stroke (185). The role of natriuretic peptides, in particular, has been validated in several stroke cohorts (24,55,195). Of note, a retrospective analysis of the WARSS study showed that elevated NT-proBNP concentrations may identify a subgroup of ischemic stroke patients without known atrial fibrillation, who may benefit more from anticoagulants than antiplatelet agents (196).

Including some of the parameters above, several studies have tried to develop risk-scoring systems to predict AF development after ischemic stroke (166,189,197–202). One of these studies evaluated predictors of AF in patients with cryptogenic stroke or TIA, composing a risk scoring system, the HAVOC score, that include 7 common clinical variables: Hypertension, Age (≥ 75), peripheral Vascular disease, Valve heart disease Obesity, Congestive heart failure, and Coronary artery disease, (169). However, with the exception of one small study (203), none of these studies analyzed predictors of AF in a clearly-defined ESUS population and none of the existing predictive scores is the output of a thorough analysis that takes, synchronously, into account every possibly related parameter, including demographics, clinical symptoms, medical history data, laboratory investigations, electrocardiographic and echocardiographic parameters as well as brain and carotid imaging findings. This is what the proposed study attempted to accomplish, i.e to

develop a diagnostic tool for the identification of ESUS patients with high or low probability of new incident AF, deriving from the analysis of all the available parameters registered in large stroke registries.

6 Methodology

We analyzed a large dataset of ESUS patients from different sites with the main objective to a) identify predictors of covert AF in ESUS patients and b) develop a prognostic score for the identification of covert AF in this population. Several parameters have been registered and included in a logistic regression model to identify independent predictors of covert AF in ESUS patients. Based on this logistic regression model, an integer-based point-scoring system have been developed. The present research is an investigator-initiated study, called Prediction of AF in ESUS (AF-ESUS) study (ClinicalTrials.gov Identifier: NCT02766205), and is supported by Pfizer through the BMS/Pfizer European Thrombosis Investigator Initiated Research Program (ERISTA).

In addition to creating a prognostic model for AF detection, several other interesting correlations have arisen between some of the recorded factors and the likelihood of AF detection or stroke recurrence.

6.1 Study population

The dataset has been derived from three high quality, prospective stroke registries: the *Acute STroke Registry and Analysis of Lausanne* (ASTRAL), the *Athens Stroke Registry* and the *Larissa Stroke Outcome Registry* (LASTRO). Eligible patients and their data have been sought both retrospectively and prospectively. In addition, other stroke registries have been also sought to identify ESUS patients which could be used to validate the observed

results and scores. The use of these registry data for research has been approved by the local ethics committees, when necessary.

6.1.1 The ASTRAL Registry

The ASTRAL registry was designed as a databank of acute ischemic stroke patients and incorporates detailed clinical and laboratory data and modern brain imaging techniques, in order to analyze underlying causes and mechanisms of ischemic stroke, to integrate clinical and radiologic data from multimodal acute stroke imaging, to follow trends of the characteristics of ischemic stroke in the geographic region, to compare the study population with similar datasets in other geographic, ethnic or racial populations and finally, to design adequately powered prevention and interventional clinical trials.

All consecutive patients that are admitted to the stroke unit and/or intensive care unit of the Centre Hospitalier Universitaire Vaudois (CHUV) in Lausanne, Switzerland with a main discharge diagnosis of acute ischemic stroke are included in ASTRAL, starting 01/01/2003. Stroke is defined as new syndrome of rapidly developing clinical symptoms and/or signs of focal disturbance of cerebral function lasting more than 24 hours, with no apparent cause other than of vascular origin, regardless if infarction was evident on cerebral radioimaging or not. ASTRAL focuses exclusively on acute ischemic stroke. Only patients being admitted within 24 hours after ischemic stroke onset (or last well-time) are included. Patients with in-hospital stroke are only included if stroke is their main pathology warranting treatment in the stroke unit and/or in the intensive care unit.

At the CHUV, virtually all patients presenting in the emergency department with a main admission diagnosis of acute ischemic stroke are admitted to and treated in the stroke unit of the Neurology service. Less than 5% of patients are treated for acute stroke in the general intensive care unit at some point of their hospitalization; these patients are also included in ASTRAL, regardless whether they passed through the stroke unit or not. Finally, recurrent acute ischemic strokes are registered in ASTRAL as new databank entry if it leads to a new hospitalization.

Patients with TIA, defined as complete disappearance of signs and symptoms within 24 hours, were excluded from ASTRAL, regardless of infarction being shown on neuroimaging or not. Patients with acute symptoms attributed to intracerebral hemorrhage (ICH), subarachnoidal hemorrhage, or cerebral sinus venous thrombosis are also excluded. Finally, ischemic stroke patients admitted later than 24 hours after initiation of symptoms were also excluded.

Collection and entry of data starts on the day of admission and further data is prospectively added during hospitalization as they become available. Most data are entered by stroke physicians, and missing data after discharge and during the 12 months follow-up are entered by stroke physicians, stroke nurses, and trained medical students. Great effort is made to obtain reliable and precise information about prestroke morbidity by interviewing patients, their relatives, stroke witnesses, ambulance drivers and primary care physicians. Old medical charts are obtained from hospital archives, primary care physicians and specialty physicians.

Patients` age, gender, ethnicity and insurance are recorded. A systematic search is performed for every patient in order to identify vascular risk factors which may be already

known or newly diagnosed. We also record previous strokes, TIAs or retinal ischemia, as well as prior medication. Finally, pre-stroke neurological status is recorded (pre-stroke NIHSS score and Rankin scale).

The exact time of onset of symptoms (or the last time that patient was reported to be well), the arrival pathways, the mode of transport to CHUV, the exact time of arrival, as well as the time intervals from stroke onset to brain imaging and to acute intervention (if performed) are all recorded in detail. Strokes were considered « unknown onset » if more than one hour had elapsed between last proof of wellbeing and time of discovery of stroke.

On admission, thorough clinical examination is performed by the admitting neurologist or neurology resident, and neurological deficits are recorded. NIHSS score is recorded systematically on admission, 6 and 24 hours after hospital arrival, upon worsening, at 7 days and at discharge. Stroke territory and topography is determined from all the information available (clinical and radiological).

Laboratory examinations (full blood count, plasma glucose, creatinine, total cholesterol, electrolytes, INR, aPTT), electrocardiographic assessment, and chest X-ray are routinely performed for all patients. Metabolic and physiological values from two time points are entered in the database: an « acute value » (first available value within the first 24 hrs after stroke onset), and a « subacute value » (first value available at 24 to 48 hours after stroke onset). Transthoracic and/or transesophageal echocardiography is performed on a as-needed basis according to a prespecified protocol. All patients get at least 24 hours continuous monitoring of vital and neurological signs, ECG, and oxygen saturation. Further specialized investigations (serum, cardiac, genetic, conventional angiography) are performed if indicated by the baseline clinical and paraclinical information.

All ischemic stroke patients undergo acute brain parenchymal imaging (CT or MRI) as soon as possible after arrival in the emergency room. Early ischemic changes, old strokes, leukoaraiosis and haemorrhagic transformations are noted. Multimodal CT imaging was the most frequently used method in the acute phase till 2018, and multimodal MRI-imaging since then Acute conventional angiography is added when intravascular treatment is likely. Arterial abnormalities are considered significant if occlusion, >50% stenosis or any signs of dissection are present.

Most patients are followed up with a second CT or MRI during the first weeks after stroke, in order to add more information about the infarct including infarct volume, hemorrhagic transformation, edema, or initially missed lesions. Follow-up subacute arterial imaging (CT angiography, MR angiography or Doppler) is performed in most patients who had an arterial pathology in the acute phase in order to investigate whether recanalization has occurred. Neuroimaging data are reviewed by stroke neurologists and neuroradiologists separately and discussed in a weekly joint session if discrepancies arise.

Patients' handicap is systematically recorded at 7 days, 3 months and 12 months after stroke, either in person at the outpatient stroke clinic or by phone by medical personnel certified in the modified Rankin score examination. In case of a suspected recurrent cerebrovascular event, confirmation is sought from the treating general physician or hospital. If a patient cannot be reached, local citizen registries are checked. Finally, when a patient passes away during the follow-up period, the cause of death is recorded according to medical records and the death certificate (204).

ASTRAL was approved in 2007 by the medical ethical committee of the University of Lausanne. Since new Swiss health research legislation came into act in 2013, analyses

are performed in anonymous fashion with a quality assurance goal, obviating the need for patient consent and ethical committee approval. Initially, data were collected in a Microsoft EXCEL datasheet. In 2009, the registry was converted to a Microsoft ACCESS database **(appendix 2)** that contains data in separate spread sheets.

6.1.2 The Athens Stroke Registry

The Athens Stroke Registry includes all consecutive patients with an acute first-ever ischemic stroke admitted in Alexandra University Hospital, Athens, Greece between June 1992 and December 2011. Patients with transient ischemic attack (TIA) or recurrent stroke are not included in the registry (15). The scientific use of the data collected in the Athens Stroke Registry was approved by the local Ethics Committee.

Detailed data were prospectively recorded including demographics, medical history and associated cardiovascular risk factors, current medication, time of stroke onset and hospital admission, duration of hospitalization, stroke characteristics, clinical findings and vital signs on admission, laboratory investigations and treatment. Stroke severity was assessed by means of the National Institute Health Stroke Scale score (NIHSS) at admission. For the study period between 1993 and 1998, NIHSS score was calculated from the Scandinavian Stroke Scale using the following formula: NIHSS score= 25.68-(0.43*Scandinavian Stroke Scale score) (205).

All patients had a 12-lead electrocardiogram (ECG) at admission. In patients on sinus rhythm, paroxysms of AF were sought by means of a) repeated ECGs during hospital stay, b) continuous ECG monitoring for 1 week or until discharge for patients treated in the

acute stroke unit, and c) 24-hours Holter ambulatory ECG monitoring in cases that AF was strongly suspected from the clinical presentation and/or brain imaging findings (e.g. multiterritorial infarcts, strokes presenting with maximum severity at onset, largely dilated left atrium) and a and b were negative.

Hypertension was defined as systolic blood pressure >140mmHg and/or diastolic blood pressure >90mmHg diagnosed at least twice before stroke or if patient was already on antihypertensives (206). Diabetes mellitus was defined if patient was already on antidiabetic drugs and/or insulin, or if fasting blood glucose level was >6.0mmol/l before stroke (207). Dyslipidemia was defined as total cholesterol concentration >6.5 mmol/l the day after admission, or if patient had a previous diagnosis of dyslipidemia (208). Coronary heart disease was assessed by questionnaire and relevant medical confirmation. Heart failure was defined according to the criteria recommended by the working group on heart failure of the European Society of Cardiology. TIA was defined as complete disappearance of signs and symptoms within 24 hours, regardless of infarction being shown on neuroimaging (209). Stroke was defined according to the World Health Organization criteria (210).

6.1.3 The LASTRO Registry

The LASTRO Registry is the prospective stroke registry of all patients with acute ischemic stroke admitted in the Department of Medicine of the University of Thessaly, which is located in the Larissa University Hospital, Larissa, Greece. It was initiated in 2013 and all patients with acute ischemic stroke are registered; on the contrary, patients with TIA

or hemorrhagic strokes are not registered. Patient data are registered in a Microsoft ACCESS file **(appendix 3)**, whereas a back-up hard copy is also kept.

Numerous parameters are registered by medical personnel involving demographics, clinical, radiological and laboratory covariates. Special focus is given in the outcome of the patients which is assessed at three months and yearly after the index stroke either at the outpatient clinic or during a telephone call with the patient or his/her proxies. Outcome endpoints assessed include functional outcome of the patient (assessed by the modified Rankin Scale score), stroke recurrence, transient ischemic attack, major hemorrhage, hemorrhagic stroke, aortic aneurysm rupture, myocardial infarction, mortality. Also, in each contact with the patient/proxies, the medication of the patient is registered with the aim to assess the adherence of the patient to the recommended medical treatment.

6.2 Definitions

We defined ESUS according to the criteria proposed by the Cryptogenic Stroke/ESUS International Working Group as a non-lacunar brain infarct in the absence of (a) extracranial/intracranial atherosclerosis causing 50% luminal stenosis in the arteries which supply the ischemic territory; (b) major-risk cardioembolic source, and (c) any other specific cause of stroke (1). All patients underwent at least 24 h of continuous cardiac monitoring, according to the Cryptogenic Stroke/ ESUS International Working Group criteria (1). The ischemic infarct was visualized with CT or MRI, according to the Cryptogenic Stroke/ESUS International Working Group criteria (1). The assessment of carotids was performed by any available imaging including ultrasound, CT-angiography, MR-angiography, and digital subtraction angiography. For pragmatic reasons, imaging of

the intracranial arteries was not required for the definition of ESUS, similar to the approach that was followed in the NAVIGATE-ESUS trial (211) and which can be justified in a Western population by the low prevalence of intracranial stenosis as a stroke mechanism (212). Patients on anti-arrhythmic drugs were not excluded from the analysis.

6.3 Data collection

A standardized form with pre-specified parameters has been used to collect and pool data of all consecutive ESUS patients that has been registered in the three above-mentioned stroke registries till 2017. For the collection of all the available data, patient's medical archives were thoroughly reviewed. Parameters included in the standardized form were:

- demographics (age, sex, ethnicity)
- patients' characteristics (height, weight, body mass index)
- presenting clinical symptoms as reported by the treating stroke physician (stroke severity, given as NIHS score on admission, aphasia, dysarthria, eye deviation, paresis, sensory symptoms, neglect, hemianopia, impaired consciousness, brainstem symptoms)
- medical history [arterial hypertension, dyslipidemia, diabetes, current smoking, coronary artery disease, peripheral artery disease, heart failure, previous stroke or transient ischemic attack (TIA), chronic obstructive pulmonary disease, history of prosthetic valve implantation, obesity, cancer, alcohol consumption, migraine]
- acute brain imaging findings as reported by board-certified consultant radiologists [brain side of infract, localization (ACA/ MCA/ PCA/ Vertebrobasilar territory),

cortical/subcortical infarct, single- or multi-territorial infarct, leukoaraiosis, brain oedema, hemorrhagic transformation, chronic infarct, ASPECTS score]

- laboratory exams on admission (glucose, hemoglobin, creatinine, white blood cells, platelets, total cholesterol, LDL cholesterol)
- electrocardiographic parameters in all available 12-lead standard ECGs performed during hospitalization for the ESUS [signs of previous MI (Q waves), atrioventricular blocks, bundle branch blocks, heart rhythm (sinus or pacemaker), Sokolov index, left ventricular hypertrophy (defined by Sokolov index >35mm or left ventricular strain), ST segment elevation, PR interval duration, QRS duration, QT duration, total duration of all inhospital ECGs (in seconds), number of supraventricular and ventricular extrasystoles / 10sec of ECG]
- parameters from in- or/and outpatient long-term cardiac monitoring [total duration of monitoring, total number of supraventricular and ventricular extrasystoles, episodes of pauses >2,5 sec, presence of supraventricular and ventricular runs (>3 consecutive beats) and their total duration]
- parameters from transthoracic or transesophageal echocardiography as reported by board-certified consultant cardiologist (left atrial diameter, left atrial volume, left ventricular ejection fraction, left ventricular diastolic dysfunction, aortic and mitral stenosis/regurgitation, left ventricular hypertrophy, left ventricular wall thickness, interventricular septum thickness, end-diastolic left ventricular internal diameter and volume, ascending aorta diameter, presence of patent foramen ovale). The presence of PFO was assessed by transthoracic echocardiography with microbubble test and, when deemed necessary by the treating physicians, by transesophageal echocardiography

- carotid imaging parameters [presence of ipsilateral or contralateral non-stenotic plaques, i.e. causing <50% stenosis, plaques' consistency (calcified/ non calcified or mixed plaques)]. These parameters were assessed by reviewing the reports of ultrasound examinations or CT angiography or magnetic resonance angiography or digital subtraction angiography. We did not review the raw images to confirm the reports.
- treatment at discharge (antiplatelet or oral anticoagulant).

6.4 Follow-Up Evaluation

Follow-up of patients from the 3 registries was systematically recorded with a structured questionnaire by medical personnel. The follow-up was performed during on-site patient visits at the outpatient clinic and/or by contact with the patient and/or the next of kin or the patient's primary physician. During the first year after stroke, patients were evaluated at 3 and 12 months after stroke. Afterwards, patient evaluation was performed on a yearly basis or at the end of the assessment period (second semester of 2017). During patient evaluation, all available information was assessed, e.g. discharge letters from hospitalizations, letters from primary physicians, and investigations performed on the outpatient setting. In case of a suspected recurrent cerebrovascular event not admitted at the 3 aforementioned hospital centres, confirmation was sought from the treating general physician or hospital. If a patient could not be reached, local citizen registries were contacted.

6.5 Outcomes

The main outcome was new incident AF during follow-up, similar to the outcome assessed in other similar studies (213). Secondary outcomes were ischemic stroke recurrence and death during follow-up. Apart from the above, we also assessed myocardial infarction, intracranial hemorrhage, and major extracranial bleeding during follow up. New incident AF was considered present if confirmed by an ECG performed for any reason including palpitations, irregular pulse on clinical examination, or by inhospital surveillance on another admission or by portable outpatient monitoring. The decision for post discharge cardiac monitoring was up to the treating physicians. Its type and duration were recorded retrospectively for this study. As concerns stroke recurrence, it was ascertained from all available information, including, the patient's medical chart and imaging. Patients who experienced >1 recurrence during the follow-up period were censored at the time of the first event.

6.6 Objectives

As already mentioned, the main objective of the study was to identify independent predictors of new incident AF and develop a predictive score for AF detection.

Howbeit, except for the main objective, in the course of analyzing the set of the collected parameters in our ESUS dataset, we also attempted:

- to assess the prognostic performance of different thresholds of left atrial diameter (LAD) for the prediction of AF, and additionally assess whether there is an association between LAD and stroke recurrence

- to assess the association between the presence of supraventricular extrasystoles (SVE) on standard 12-lead ECG and the rate of AF detection, stroke recurrence or death during follow-up,
- to assess the association between the presence of ipsilateral non-stenotic carotid plaques and the rate of AF detection, stroke recurrence or death during follow-up,
- to assess the prevalence and degree of overlap of potential embolic sources (PES) in patients with ESUS and assess differences in stroke recurrence rates between PES.
- to investigate potential embolic sources in ESUS using a data-driven, machine learning method, and explore variation in stroke recurrence between clusters
- to compare the baseline characteristics between mild and severe ESUS and assess outcomes of patients with severe ESUS.
- to assess the RoPE score (Risk of Paradoxical Embolism) distribution in our ESUS population and investigate the rate of stroke recurrence and new incident AF during follow-up according to PFO status and RoPE score.
- to develop and externally validate a score for the identification of ESUS patients at high risk for stroke recurrence
- to assess the performance of the HAVOC score in our ESUS dataset.

6.7 Statistical Analysis

For each parameter we examined, in order to assess the association between the parameter and the main (AF) or the secondary outcomes (stroke recurrence and death), we performed multivariate stepwise regression with forward selection of covariates including most of the data collected and described in detail in section 6.3. More specifically, the

selected covariates included demographics (age, sex), presenting clinical symptoms (stroke severity, aphasia, dysarthria, eye deviation, paresis, sensory symptoms, neglect, hemianopia, impaired consciousness, brainstem symptoms), medical history (hypertension, dyslipidemia, diabetes, smoking, coronary artery disease, peripheral artery disease, heart failure, previous stroke or transient ischemic attack, obesity, cancer, alcohol consumption), acute brain imaging findings (localization, lateralization, leukoencephalopathy, brain oedema, hemorrhagic transformation, chronic stroke, ASPECTS score) electrocardiographic parameters (atrioventricular blocks, ST elevation, PR interval, ventricular extrasystoles, Sokolov index, echocardiographic parameters (left atrial diameter, left atrial volume, ejection fraction, aortic and mitral stenosis/regurgitation, left ventricular wall thickness, interventricular septum thickness, end-diastolic volume), carotid ultrasound parameters (stenosis and plaque in the arterial tree of the ischemic and the nonischemic territory) and treatment at discharge (antiplatelet or oral anticoagulant). In order to estimate the 10-year cumulative probability of the main or the secondary outcomes, we used the Kaplan-Meier product-limit method. In order to calculate the sensitivity, specificity, positive prognostic value, negative prognostic value and Youden's J statistic of the presence of a specific parameter or of different thresholds of a score or a variable to predict new incident AF, we used the following equations: Sensitivity: a/(a+b), specificity: d/(c+d), positive predictive value: a/ (a+c), negative predictive value: d/(b+d) and Jouden's J statistic: sensitivity+specificity-1, where a is the number of patients having the specific parameter or patients above a specific threshold who were diagnosed with AF, b is the number of patients not having the specific parameter or patients below a specific threshold who were diagnosed with AF, c is the number of patients having the specific parameter or patients above a specific threshold who were did not have a diagnosis of new incident AF

and d is the number of patients not having the specific parameter or patients below the specific threshold who did not have a diagnosis of new incident AF.

Continuous covariates are summarized as median and interquartile range (IQR) or mean and standard deviation (SD); nominal variables are given as count and absolute percentages. For the multivariate analyses, the level of significance was set at 5%. Associations are presented as hazard ratios (HR) with their corresponding 95% confidence intervals (95% CI). For patients lost during follow-up, survival data were censored at the last time known to be alive. For the outcome of stroke recurrence, patients who experienced >1 recurrences during the follow-up period were censored at the time of the first event. Differences in Kaplan-Meier curves were evaluated with the log-rank test and the level of significance was set at 5%. Statistical analyses were performed with the Statistical Package for Social Science (SPSS, Inc., version 20.0 for Windows, Chicago, IL). Investigators, involved in the statistical analysis, complied with the authorship guidelines in the Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals (icmje.org/icmje-recommendations.pdf) established by the International Committee of Medical Journal Editors. Reports of the analyses follow the STROBE statement guidelines. In case a different statistical method was used for some of the analyzes, this is referred separately in the corresponding paragraph. Statistical analysis performed for the development of the multivariate model for the prediction of atrial fibrillation is described in detail in paragraph 7.3.

7 Results

7.1 Population's baseline characteristics

7.1.1 Demographics and comorbidities

Overall, 884 consecutive ESUS patient, registered in the 3 pooled registries, were enrolled in our dataset (43.3% women). From them, 573 patients (64,8%) were registered in the stroke registry of Lausanne (ASTRAL), 275 patients (31,1%) in the Athens stroke registry and 36 patients (4,1%) in the stroke registry of Larissa (LASTRO). Their median age was 67 years, interquartile range [IQR] 54.5–77) and they were followed for a median of 23.7 months, corresponding to an overall follow-up period of 2,899 patient-years. Among these patients, 91.6% were discharged on antiplatelet treatment. The baseline characteristics of these patients as concerns demographics and their comorbidities are summarized in the **table 3.**

Table 3 : *Overall population's demographics and comorbidities*

Variable	**Overall population (N = 884)**
Demographics	
Age (years)	67.0 (54.5-77)
Female sex	383 (43.3%)
Comorbidities	
Arterial hypertension	546 (61.8%)
Dyslipidemia	585 (66.2%)
Diabetes mellitus	161 (18.2%)
Current smoking	339 (38.3%)
Coronary artery disease	132 (14.9%)
Peripheral artery disease	25 (2.8%)
Previous stroke or TIA	149 (16.9%)
Cancer	82 (9.3%)
Previous myocardial infarction	106 (12.0%)

7.1.2 ESUS severity

The assessment of ESUS severity in our population was made using the National Institutes of Health Stroke Scale (NIHSS) score on admission. From the 884 registered ESUS patients, information about NIHSS score on admission was available in 772 patients with a mean age of 64 ±17 years, from which 42,6% were women. The median NIHSS score on admission was 6 (interquartile range; IQR: 3–12) while the distribution of baseline NIHSS score in the population is presented in **figure 1.** Using the median NIHSS score of the cohort as a threshold between severe and mild ESUS, 358 patients were classified as mild ESUS (admission NIHSS < 6) and 414 as severe ESUS (admission NIHSS ≥ 6). Analysing and comparing the baseline characteristics of the two groups (mild and severe ESUS patients), which are presented in **Table 4**, the proportion of women was higher among patients with severe ESUS compared with mild (48.6% vs. 35.8% respectively, $p < 0.001$), while no significant differences were observed in comorbidities and cardiovascular risk factors. Oral anticoagulants were more frequently prescribed in patients with severe ESUS compared with mild (5.6% vs. 4.2% respectively, $p = 0.02$). In the multivariable logistic regression, only female sex (OR: 1.72, 95%CI: 1.27–2.33) was independently associated with severe ESUS.

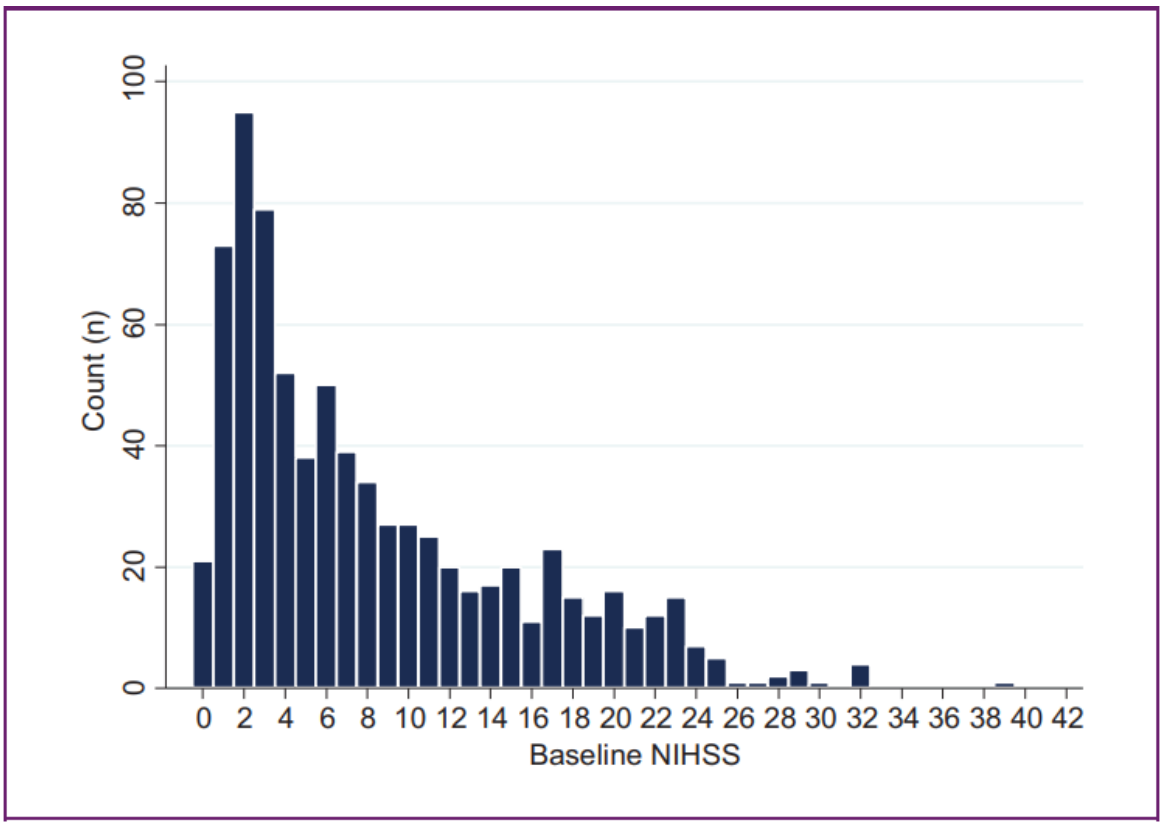


Figure 1: *Distribution of baseline NIHSS score in the overall cohort.*

Table 4: *Baseline characteristics of patients according to stroke severity*

	Severe ESUS (n = 414)	**Mild ESUS (n = 358)**	**p value**
Female sex	201 (48.6%)	128 (35.8%)	<0.001
Age (y), mean ± SD	64.9 ± 16.4	63.6 ± 16.7	0.138
NIHSS on admission (median (IQR))	12 (8-18)	2 (1-4)	<0.001
Hypertension	263 (63.5%)	214 (59.8%)	0.285
Dyslipidemia	272 (65.7%)	241 (67.3%)	0.635
Diabetes	76 (18.4%)	63 (17.6%)	0.784
Heart Failure	11 (2.7%)	12 (3.4%)	0.571
Smoking	166 (40.1%)	135 (37.7%)	0.498
Previous Ischemic Stroke /TIA	63 (15.2%)	61 (17%)	0.492
Antiplatelet at discharge	376 (90.8%)	339 (94.7%)	0.04
Anticoagulant at discharge	42 (10.1%)	20 (5.6%)	0.02

7.1.3 Important echocardiographic, electrocardiographic, and imaging characteristics

7.1.3.1 Left atrial Dilatation

Among the 884 patients with ESUS, information about left atrial diameter (LAD) was available at the echocardiography report in 676 (76.5%) patients (median age 66.5 years, 41.4% women). The median LAD was 39 mm (interquartile range: 34–43) while the proportion of patients with LAD above different thresholds are presented in **table 5**. Comparing the baseline characteristics of patients with LAD below and above the threshold of 40mm, which is the threshold widely used in clinical practice to describe left atrial dilatation, patients with LAD >40 mm were older and had more frequently hypertension, diabetes and coronary artery disease compared to patients with LAD ≤40 mm (**table 6**)

***Table 5**: Proportion of patients with different LAD thresholds*

LAD (mm)	*>37*	*>38*	*>39*	*>40*	*>41*	*>42*	*>43*	*>44*	*>45*	*>46*	*>47*	*>48*	*>49*	*>50*	*>51*	*>52*	*>53*	*>54*
Proportion of patients above this threshold (%)	56,4	50,7	46,0	39,1	35,2	28,3	22,5	17,6	13,0	10,1	9,0	7,1	6,2	3,4	2,5	1,6	1,5	1,0

***Table 6** : Baseline characteristics of patients by LAD threshold of 40mm*

	LAD ≤40mm (n= 405)	**LAD >40mm (n= 271)**	**P-value**
Female sex	180 (44.4)	100 (36.9%)	0.06
Age (y) median (IQR)	61 (49-72)	72 (64-80)	<0.001
Hypertension	209 (51.6%)	205 (75.6%)	<0.001
Diabetes	58 (14.3%)	66 (24.4%)	0.001
Smoking	120 (29.6%)	68 (25.1%)	0.06
Coronary artery disease	47 (11.6%)	62 (23.0%)	<0.001
Previous stroke	54 (13.3%)	45 (16.6%)	0.12
Antiplatelet at discharge	374 (94.0%)	243 (91.7%)	0.23
Anticoagulant at discharge	30 (7.5%)	23 (8.7%)	0.41

7.1.3.2 Supraventricular Extrasystoles

As described thoroughly in section 6.3, during data collection, we measured the number of SVEs in all available standard 12-lead ECGs during the hospitalization for ESUS as well as the number of SVE per 10 seconds of all available ECG. Among the 853 patients with ESUS (median age: 67 years, 43.0% women) and available ECG for evaluation in their medical charts, 226 (26.5%) patients had at least 1 SVE at the standard 12-lead ECGs performed during hospitalization, while the mean number of ECGs performed per patient was 3.7 ± 3.9 (median of 3). According to the number of SVE per 10 seconds of all available ECG, patients were categorized in 4 groups: with no SVE, with greater than 0-1 SVEs, with greater than 1-2 SVEs, and with greater than 2 SVEs. For example, a patient who had 40 seconds of ECGs during hospitalization with a total of 2 SVEs would be classified in the first group (as he/she would have 0.5 SVEs per 10 seconds), whereas a patient who had 60 seconds of ECGs during hospitalization with a total of 12 SVEs would be classified in the second group (as he/ she would have 2 SVEs per 10 seconds). The baseline patient characteristics in the 4 SVE groups are summarized in **Table 7**. Comparing the baseline characteristics between the 4 groups, patients with no SVEs were younger and had less frequently hypertension, diabetes, and coronary artery disease (**Table 7)**.

Table 7: *Baseline patient characteristics by the number of SVEs per 10 seconds of ECG*

	No SVE (n= 627)	>0-1 SVE (n= 111)	>1-2 SVE (n= 57)	>2 SVE (n= 58)	P-value
Female sex	274 (43.7)	40 (36.0)	18 (31.6)	35 (60.3%)	0.06
Age(y), median (IQR)	65 (51.5-76)	71 (63-80)	71 (65-78)	73.5 (64-7)	<0.001
Hypertension	359 (57.3%)	81 (73.0%)	40 (70.2%)	48 (82.8%)	<0.001
Diabetes	104 (16.6%)	22 (19.8%)	17 (29.8%)	15 (25.9%)	0.001
Smoking	264 (42.1%)	36 (32.4%)	15 (26.3%)	17 (29.3%)	0.06
Coronary artery disease	71 (11.3%)	28 (25.5%)	15 (26.3%)	14 (24.6%)	<0.001
Previous stroke	113 (18.0%)	18 (16.2%)	4 (7.0%)	8 (13.8%)	0.12
Number of ECGs	2 (1-3)	5 (4-7)	5 (4-7)	6 (5-15)	<0.001
Antiplatelet at discharge	584 (93.9%)	93 (85.3%)	51 (96.2%)	52 (96.3%)	0.25
Anticoagulant at discharge	43 (6.9%)	15 (13.8%)	3 (5.7%)	3 (5.6%)	0.37

7.1.3.3 Nonstenotic carotid plaques

Information about the presence or absence of ipsilateral nonstenotic carotid plaques was available in 777 patients (43.3% women) with a median age of 67 years (interquartile range 54–77). The presence of ipsilateral nonstenotic carotid plaques was reported in 341 (38.6%) patients. Looking the baseline characteristics of patients, which are summarized in **table 8**, patients with ipsilateral nonstenotic carotid plaques were older, more frequently had arterial hypertension, dyslipidemia, and previous stroke, and were more frequently active smokers in comparison with patients without plaques.

Table 8: *Baseline characteristics of patients with and without ipsilateral nonstenotic carotid plaques*

	Carotid Plaques present (n=341)	Carotid Plaques absent (n=436)	P value
Female sex	156 (45.7%)	180 (41.3%)	0.21
Age (y), median (IQR)	73 (64-80)	64 (50-74)	<0.001
Hypertension	257 (75.4%)	242 (55.5%)	<0.001
Dyslipidemia	266 (78%)	254 (58.2%)	<0.001
Diabetes	62 (18.2%)	84 (19.3%)	0.71
Smoking	149 (43.7%)	148 (33.9%)	<0.01
Coronary artery disease	44 (12.9%)	79 (18.2%)	0.06
Previous stroke	82 (24%)	51 (11.7%)	<0.01
Antiplatelet at discharge	321 (95.3%)	384 (90.1%)	<0.001
Anticoagulant at discharge	16 (4.7%)	44 (10.3%)	<0.01

7.1.3.4 Patent foramen ovale and RoPE score

Among the 884 registered patients with ESUS in the 3 pooled registries, 455 had available information about the presence or absence of PFO. When compared with patients with unknown PFO status, patients with known PFO status were younger, had less vascular risk factors, less atherosclerosis, and higher RoPE score.

From the 455 ESUS patients with known PFO status, 288 were investigated by transthoracic echocardiography with microbubble test and 167 with transesophageal echocardiography. Their median age was 59 years, 41% were females, and PFO was present in 40% (n=184) of patients. Atrial septal aneurysm was detected in 32% of patients (n=121), 58.7% of those with PFO, and 10.8% of those without. Baseline features of patients with and without PFO are displayed in **Table 9**. The distribution of the RoPE score values in patients with known PFO status and the number of patients with PFO in the groups

with low Rope score values (Rope score :0-6) and high Rope score values (Rope score :7-10) are presented in **table 10.** The PFO was closed in 68 patients (36%). Their median RoPE score of 8 (interquartile range, 7–9) was significantly higher than the score of patients whose PFO was not closed (median 6, interquartile range, 4–7, P=0.000).

***Table 9**: Baseline characteristics of patients with known PFO status*

Variables	Total (n=455)	PFO absent (n=271)	PFO present (n=184)	P value
Demographics and risk factors				
Age (y) median (IQR)	59.2 (45.9–72.2)	64.7 (53.1–77)	49 (36.8–63.7)	0.000
Female sex	186 (40.9%)	102 (37.6%)	84 (45.6%)	0.108
Arterial hypertension	235 (51.6%)	179 (66%)	56 (30.4%)	0.000
Hypercholesterolemia	296 (65%)	192 (70.8%)	104 (56.5%)	0.002
Diabetes	62 (13.6%)	52 (19.2%)	10 (5.4%)	0.000
Smoking	200 (44%)	129 (47.6%)	71 (38.6%)	0.071
Coronary artery disease	44 (9.7%)	36 (13.3%)	8 (4.3%)	0.003
Previous stroke or TIA	78 (17.1%)	56 (20.7%)	22 (12%)	0.022
BMI	25 (23–29)	26 (24–30)	24 (22–28)	0.000
Stroke characteristics and radiological features				
Baseline NIHSS	6 (2–12)	6.5 (3–13)	5 (2–11.6)	0.033
Lesion topography				0.000
Deep	41 (9.1%)	33 (12.3%)	8 (4.3%)	
Superficial	335 (74%)	204 (75.8%)	131 (71.2%)	
Infratentorial	77 (17%)	32 (11.9%)	45 (24.5%)	
Acute lesions in multiple territories	18 (4%)	15 (5.5%)	3 (1.6%)	0.064
Previous ischemic lesion	85 (18.7%)	64 (23.6%)	21 (11.4%)	0.002
Leukoaraiosis	67 (14.7%)	54 (19.9%)	13 (7.1%)	0.000
Atherosclerotic plaque in ischemic territory	165 (44.1%)	131 (53%)	34 (26.8%)	0.000
Stenosis >50% in nonischemic territory	37 (8.2%)	29 (10.8%)	8 (4.4%)	0.024
Atherosclerotic plaque in nonischemic territory	185 (41.3%)	146 (54.5%)	39 (21.7%)	0.000
Acute laboratory				
Blood glucose, mg/dL	111.6 (98–131.4)	111.6 (99–137.5)	109.8 (97.2–124.2)	0.066
Hemoglobin, g/L	14.2 (13.2–15.2)	14.1 (13.2–15)	14.4 (13.3–15.3)	0.384
White blood cells, 103/μL	8.1 (6.5–10.1)	81 (6.5–9.7)	8.0 (6.6–10.8)	0.478
Total cholesterol, mg/dL	200 (166.3–231.7)	197.2 (166.3–229.7)	201.1 (168.9–232)	0.810
LDL cholesterol, mg/dL	108 (89–143.2)	108 (85–139)	115 (89–147.7)	0.415
Echocardiography data				
Left atrial diameter	36 (32–40)	37 (32.7–42.3)	34 (30–38)	0.000
Left ventricular hypertrophy	76 (16.9%)	70 (25.9%)	6 (3.4%)	0.000
Interventricular septum hypertrophy	94 (21%)	83 (31%)	11 (6.2%)	0.000
Atrial septal aneurysm				0.000
Absent	235 (62%)	178 (84%)	57 (34.1%)	
Present	121 (31.9%)	23 (10.8%)	98 (58.7%)	
Information not available	23 (6.1%)	11 (5.2%)	12 (7.2%)	
Discharge medications				
Antiplatelets	417 (92.3%)	251 (93.3%)	166 (90.7%)	0.404
Anticoagulants	35(7.7%)	17 (6.3%)	18 (9.8%)	0.233

***Table 10**: distribution of the RoPE score values in patients with known PFO status*

RoPE score	No. of patients with known PFO status	No. of patients with PFO
0-3	108	84*
4	90	
5	56	
6	56	
7	56	95*
8	51	
9-10	38	

* PFO was closed in 12 and 56 patients, respectively.

7.1.4 Potential Embolic Sources (PES)

7.1.4.1 Prevalence and overlap of PES

In order to better analyze our population's characteristics, we tried, based on the recorded data, to estimate the prevalence as well as the degree of coexistence of potential embolic sources (PES) in the study's population. Patients were categorized in ≥1 groups according to the PES that was/were identified. In accordance with the theoretical background, analyzed in detail in chapter 7, we categorized PES as follows: atrial cardiopathy (AC), AF diagnosed during follow-up, arterial disease, LV disease, cardiac valvular disease, PFO, and cancer. When >1 PES was identified in a single patient, the patient was categorized in all applicable PES groups. Hence, the overall sum of the number of patients (calculated by adding the number of patients in each PES group) is higher than the total number of patients in our population. On the basis of previously published associations with the risk of stroke, AC was diagnosed if the echocardiogram reported left atrial dilatation or increased left atrial diameter (>38 mm for women and >40 mm for men) (214) or if supraventricular extrasystoles were present at the 12-lead ECGs performed during hospitalization (215). We diagnosed arterial disease in case of presence of any

ipsilateral atherosclerotic carotid plaque causing luminal stenosis <50% (216) of or aortic arch atherosclerosis (74) based on the imaging reports. LV disease was diagnosed if low LV ejection fraction (<35%) LV hypertrophy or left-sided heart failure was reported at the echocardiogram, or if LV hypertrophy was identified at the ECG (Sokolow index ≥35 mm). We diagnosed cardiac valvular disease if moderate-to-severe stenosis or regurgitation of the mitral or aortic valve was reported at the echocardiogram.

All the necessary information for the inclusion in one of the aforementioned PES groups was available in 800 patients (43.1% women; median age, 67.0 years). The baseline characteristics of patients by PES group are summarized in the **Table 11**. The prevalence of the classic cardiovascular risk factors did not have major differences across different PES, except for patients with PFO who were younger and had a lower prevalence of arterial hypertension, dyslipidemia, and previous stroke.

***Table 11**: Baseline Characteristics and Outcomes of Patients per PES group*

Variable	Atrial fibrillation (n=120)	Atrial Cardiopathy (n=360)	Arterial Disease (n=388)	Left Ventricular Disease (n=435)	Cardiac Valvular Disease (n=69)	Patent foramen ovale (n=170)	Cancer (n=74)	p-value
Female sex	57 (47.5)	142 (39.4%)	178 (45.9%)	185 (42.5%)	35 (50.7%)	75 (44.1%)	36 (48.6%)	0.49
Age (y) median (IQR)	73.7 (65.3-79.0)	72.0 (63.3-79.3)	72.2 (64-80)	72.0 (64-80)	74.2 (67-81)	48.6 (35-61)	74.5 (69-81)	0.00
NIHSS	5.0 (2.0-9.0)	6.0 (3-13)	7.0 (2-11)	6.0 (2-10)	7.0 (2-12)	5.0 (1-10)	7.0 (4-10)	0.40
Hypertension	98 (81.7%)	269 (74.7%)	289 (74.5%)	332 (76.3%)	57 (82.6%)	53 (31.2%)	57 (77.0%)	0.00
Dyslipidemia	73 (60.8%)	241 (66.9%)	300 (77.3%)	307 (70.6%)	46 (66.7%)	96 (56.5%)	57 (77.0%)	0.00
Diabetes	32 (26.7%)	84 (23.3%)	68 (17.5%)	98 (22.5%)	9 (13.0%)	10 (5.9%)	16 (21.6%)	0.65
Smoking	41 (34.2%)	121 (33.6%)	179 (46.1%)	167 (38.4%)	28 (40.6%)	64 (37.6%)	31 (41.9%)	0.44
Coronary artery disease	27 (22. %7)	76 (21.2%)	53 (13.7%)	69 (15.9%)	9 (13.0%)	7 (4.1%)	10 (13.5%)	0.21
Previous stroke	21 (17.5%)	59 (16.4%)	89 (22.9%)	83 (19.1%)	19 (27.5%)	19 (11.2%)	14 (18.9%)	0.00
Antiplatelet at discharge	112 (94.9%)	323 (91.8%)	370 (96.1%)	396 (93.0%)	60 (89.6%)	154 (91.1%)	70 (94.6%)	0.36
Anticoagulant at discharge	5 (4.2%)	31 (8.8%)	19 (4.9%)	35 (8.2%)	6 (9.0%)	16 (9.5%)	5 (6.8%)	0.28
Stroke recurrence*	25.20	23.62	22.42	23.90	21.70	17.91	20.61	0.37

*(Per 100 patient-years). All comparisons were performed using Cochran-Mantel-Haenszel x^2 test.

The 5 most prevalent PES were LV disease, arterial disease, AC, PFO, and AF, which were present in 54.4%, 48.5%, 45.0%, 21.3%, and 15.0% of patients, respectively. Most patients (65.5%) had >1 PES, whereas only 29.7% and 4.8% of patients had a single or no PES, respectively. In 31.1% of patients, there were ≥3 PES present. On average, each patient had 2 PES (median, 2). The prevalence of each PES and the degree of their overlap is summarized in **Figure 2.** To enhance the visualization of the prevalence of PES and the degree of their overlap, we used UpSet (Caleydo, https://cale ydo.org) to draw a matrix layout **(Figure 2).** The plot has 7 rows, each one of which corresponds to a specific PES, as described in the plot legend. Cells may be either empty (indicating absence of the specific PES) or filled (indicating presence of the specific PES). Each column corresponds to a specific combination of PES. The numbers in the plot correspond to the proportion of patients in the overall population with a specific PES (for the numbers shown at the rows) or with a specific combination of PES (for the numbers shown at the columns).

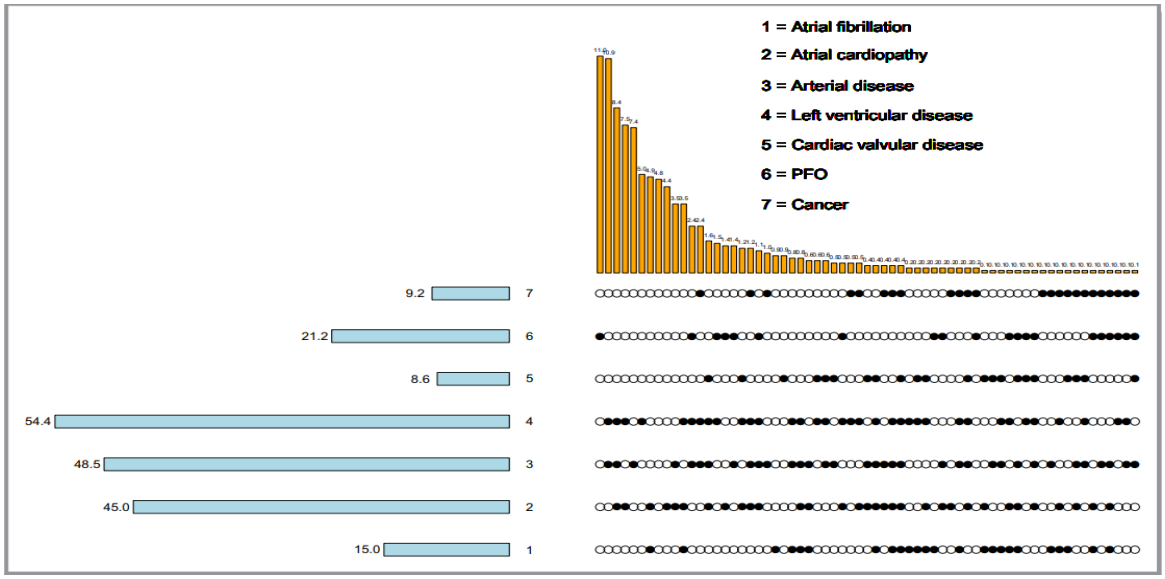


***Figure 2**: Prevalence of potential embolic sources (PES) and degree of their overlap*

7.1.4.2 Data-driven machine-learning analysis of potential embolic sources

Except for characterizing our population, using prespecified groups of PES, we also attempted to investigate potential sources of embolism in our ESUS patients using a data-driven, machine-learning analytical method.

Clustering algorithms, a common "unsupervised" machine-learning, can be used to identify groups (clusters) of similar individuals based on the sum of the combined values of their measured characteristics (217). In hierarchical clustering, the results are easily reproducible and this process is fixed once clusters are assigned, so participants cannot be reclassified into a different cluster. This contrasts with standard regression methods, which is used to identify associations between response and explanatory variables. This belongs to "supervised" learning which can be used for multiple testing to determine significant differences between groups, which need to be specified a priori. Each test is independent of the other tests, which results in groups, which are only relevant to the particular variable tested. Clustering takes into account all variables, providing a way to holistically represent the entirety of the data collected (218). This process, therefore, is extremely advantageous for exploring the potential underlying etiology in particularly heterogeneous diseases, like ESUS.

The clustering methods utilized all baseline features in our dataset (demographics, lifestyle factors, clinical symptoms/signs during the qualifying ESUS, comorbidities, biometrics, biomarkers, vascular imaging, brain imaging, electrocardiogram and echocardiography). In order to identify groups of patients with similar characteristics (i.e. clusters), we used a combined k-means and hierarchical agglomerative approach to generate clusters – called hierarchical *k*-means clustering (219). This process allows for the

k-means based approach to accelerate or speed up a traditional *k*-means algorithm in both training and query phases, which allows for a much larger number of centroids to be used, which in turn leads to much better learning (219). In this process, we pick some *k* to be the branching factor, which defines the number of clusters at each level of the clustering hierarchy. We then cluster the set of points into *k* clusters using a standard *k*-means algorithm. Finally, we recursively clustered each sub-cluster until we determine a small, fixed number of points. Using all the baseline data provided from ESUS patients, the algorithm therefore could assign each individual into a unique cluster.

To determine the optimum number of clusters, we used a combined approach using 30 different clustering indices, which includes common methods including "elbow", "average silhouette", or "gap statistics". The optimal number of clusters were determined from the highest frequency of selection from all 30 indices (220). To visualize the clustering process, we generated a dendrogram (a tree diagram) to illustrate the arrangement of the clusters produced (221). Each branching creates a unique participant cluster, with the size of the clusters determined by the height of the branches. Separately, we also conducted a principle components analysis (PCA) by plotting the first two principle components on a coordinate to observe the clusters between each ESUS patient by his/her respective assigned cluster group. These principal components were derived using the orthogonal transformation (eigenvectors and eigenvalues) to reduce down the dimensionality of the original data, from all the clinical features collected on ESUS patients. Clustering analyses and data visualisation tools were conducted using statistical software R using packages *cluster, NbClust, factoextra, dendxtend* and *ggplot2*. Comparisons across clusters were conducted using the non-parametric Kruskal-Wallis test for continuous variables and χ^2

tests for categorical variables (222,223). Prior to the clustering analysis, data which were missing-at-random were imputed using multiple imputation using chained equations (224).

We further profiled each cluster by determining the prevalence of each PES (as defined in paragraph 9.1.4.1) within each cluster. Patients were also categorized by the number of PES: 0-1 PES, 2 PES, or ≥3 PES. To quantify the contribution of each PES to each cluster, we applied logistic regression to determine the association between each PES with the derived cluster. In this analysis, the PES was the exposure variable and the cluster grouping was the outcome variable (coded as 1 – belonging to the cluster, or coded as 0 – belonging to other clusters). All models were adjusted for sex, age, dyslipidemias, diabetes mellitus, smoking, coronary artery disease, and National Institute of Health Stroke Scale (NIHSS) score at admission. The PES in each cluster were then ranked by significance and by the effect size, with 95% confidence intervals provided. In this way, we were able to "profile" each cluster and associate them to specific PES.

In a total of 800 patients (43,3% woman, median age: 67 years), from 30 clustering indices, it was found that the optimal number of clusters is 4 **(Figure 3)**. The arrangement of the 4 clusters during the clustering process is illustrated at the dendrogram **(figure 4)**. The principal components analysis identified that 82% of all principal components were needed to explain 100% variation of the original ESUS data **(figure 5a)**, which suggests that there is substantial heterogeneity between ESUS patients in clinical features, as a high number of principal components are needed to explain significant variation of the original data. By plotting the first two principal components which only explains up to 16% of the variation in the original data, visual separation can be seen between clusters from the hierarchical clustering process **(figure 5b).** Cluster sizes were as follows: 44 patients

(5.5%) in cluster 1, 149 patients (18.6%) in cluster 2, 430 patients (53.8%) in cluster 3, and 177 patients (22.1%) in cluster 4. There was overlap between cluster 1 and cluster 2. However, clusters 2, 3, and 4 all remained quite distinct, with a large degree of separation and very little overlap.

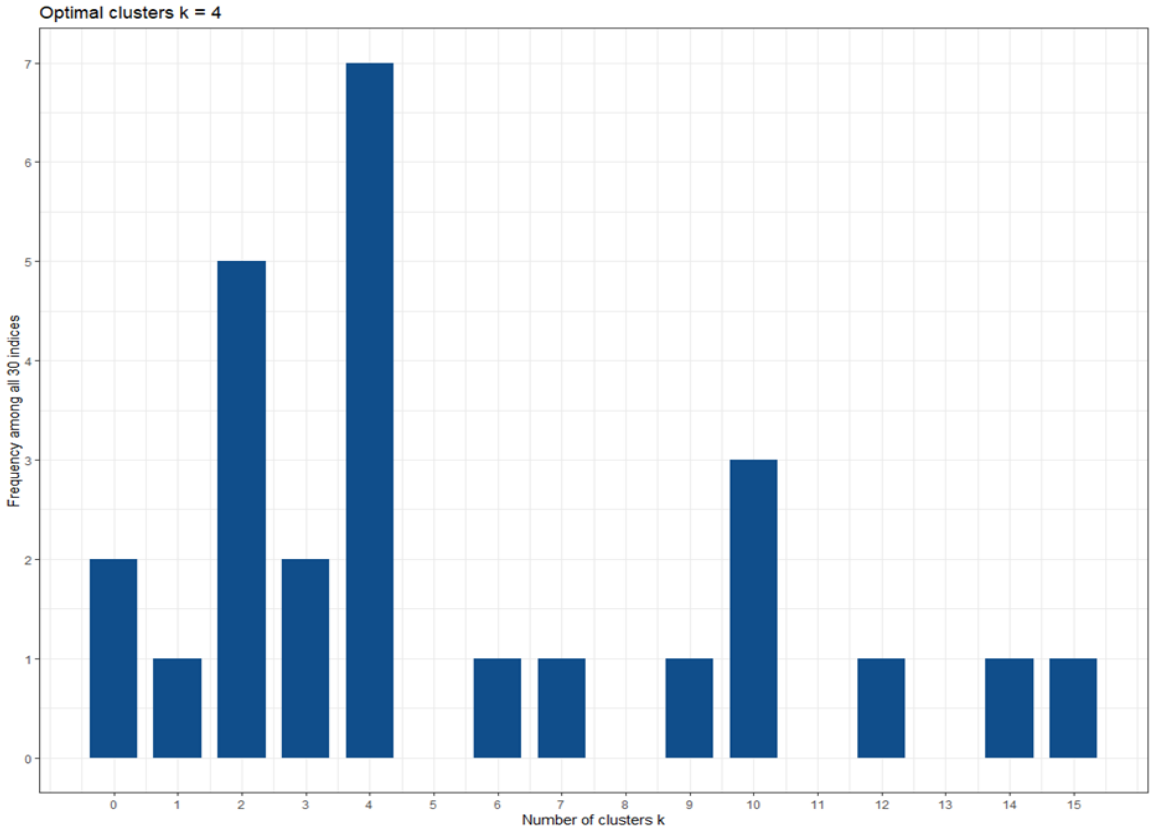


Figure 3: *Optimal number of clusters by frequency among 30 clustering indices.*

The figure indicates that 7 clustering indices identified the optimal number of clusters was 4, which was the highest selection frequency among all indices.

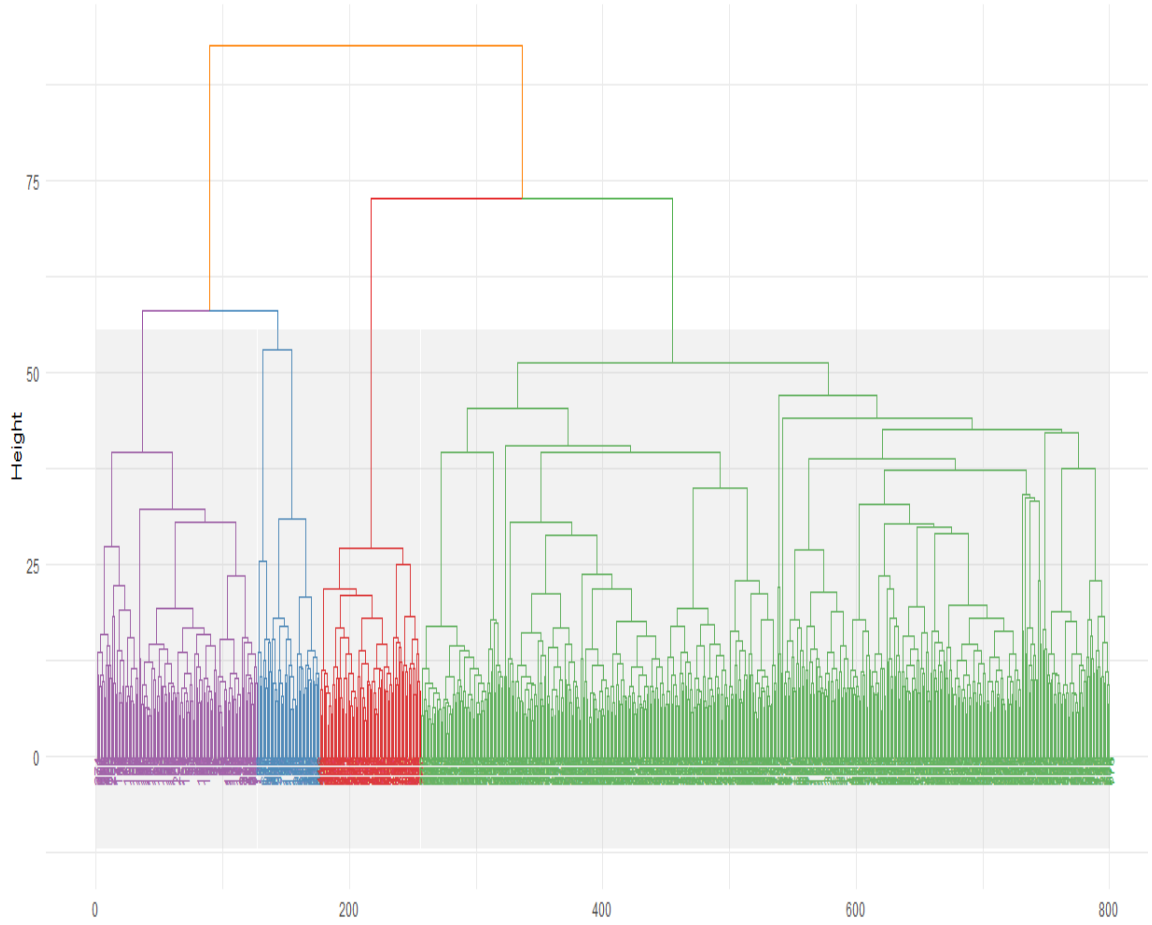


Figure 4: *Dendrogram - arrangement of the clusters produced by the hierarchical k-means clustering analysis*

Each branching creates a unique participant cluster, with the size of the clusters determined by the height of the branches. The four clusters are presented by the colored regions on the dendrogram.

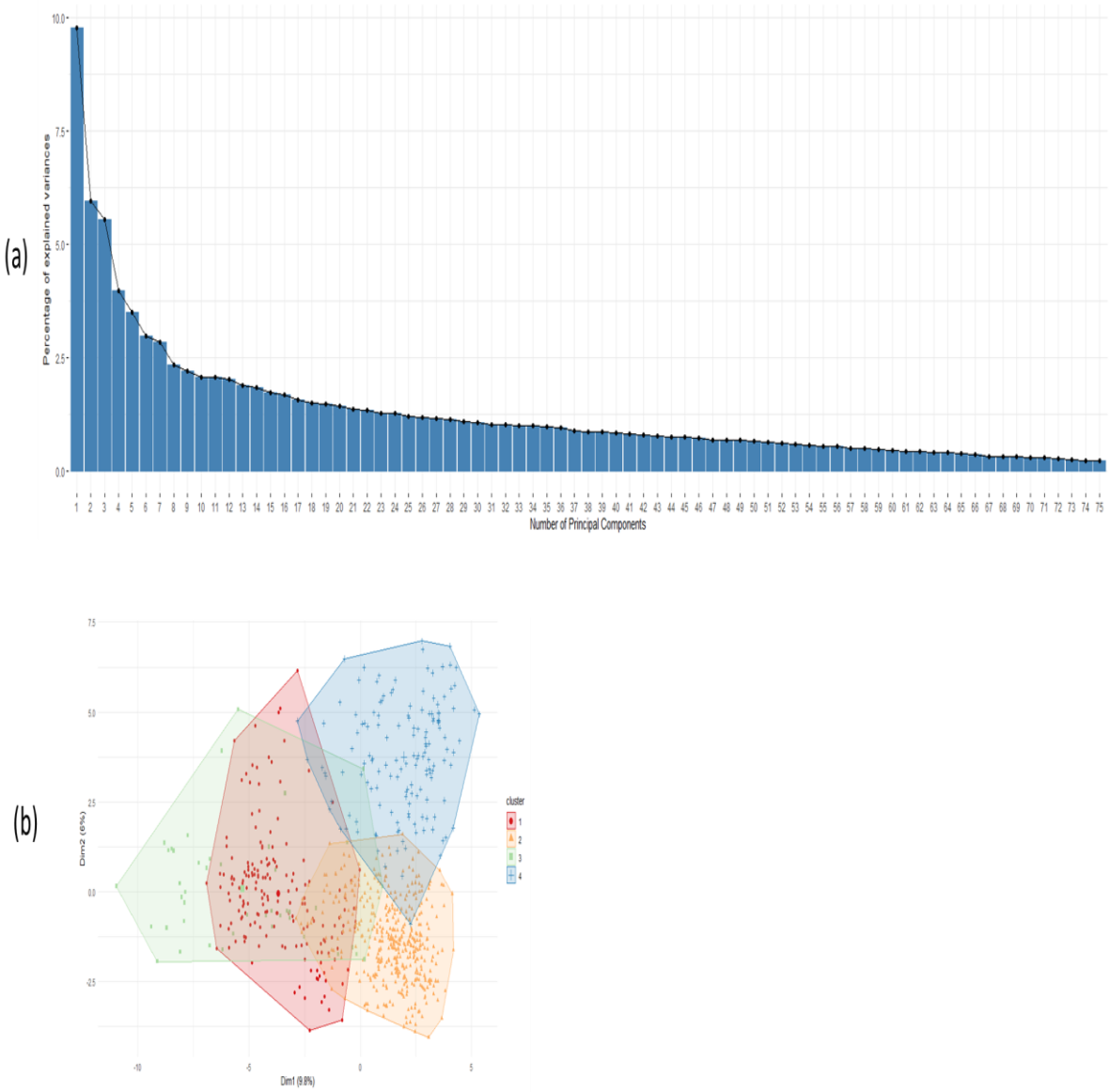


Figure 5: *Principal components analysis of collected baseline clinical features*

(a) A total of 75 principal components are needed to explain nearly 100% of variance of the data which suggests high heterogeneity between patients. (b) Plotting the first two principal components shows separation of the cluster groupings derived from hierarchical k-means clustering.

The baseline characteristics of the patients in the four clusters are summarized in **Table 12**. There were significant differences between clusters in terms of gender, baseline

age, NIHSS at admission, hypertension, diabetes mellitus, coronary artery disease, previous stroke and antithrombotic treatment at discharge.

The prevalence of each PES stratified by cluster is summarized in **Table 13**. There were significant differences between clusters in the prevalence of atrial fibrillation, atrial cardiopathy, arterial disease, left ventricular disease, PFO and cancer. Left ventricular disease was most prevalent in cluster 1 (100%). PFO was most prevalent in cluster 2 (38.9%). Arterial disease was most prevalent in cluster 3 (57.7%). Atrial cardiopathy were most prevalent in cluster 4 (100%).

Table 12: *Baseline characteristics and outcomes of patients, stratified by cluster*

		Cluster 1 (n=44)	Cluster 2 (n=149)	Cluster 3 (n=430)	Cluster 4 (n=177)	*p*-value
Age, years	Median (IQR)	67 (62–75)	62.9 (46.7–74.6)	66 (51.7–76.8)	71 (64–79)	<0.001
Female sex	n (%)	14 (32.8)	51 (34.2)	205 (47.7)	75 (42.4)	0.013
NIHSS score	Median (IQR)	9 (3–21)	3 (2–6)	7 (3–14)	6 (3–13)	<0.001
Hypertension	n (%)	30 (68.2)	80 (53.7)	250 (58.1)	136 (76.8)	<0.001
Dyslipidemia	n (%)	26 (59.1)	92 (61.7)	294 (68.4)	113 (63.8)	0.319
Diabetes mellitus	n (%)	19 (43.2)	21 (14.1)	66 (15.4)	42 (23.7)	<0.001
Smoking	n (%)	16 (36.4)	53 (35.6)	183 (42.6)	57 (32.2)	0.087
Coronary artery disease	n (%)	13 (29.6)	17 (11.4)	41 (9.53)	47 (26.6)	<0.001
Previous stroke	n (%)	3 (6.8)	21 (14.1)	83 (19.3)	21 (11.9)	0.039
Antiplatelet at discharge	n (%)	32 (72.7)	137 (92.0)	402 (93.5)	161 (91.0)	<0.001
Anticoagulant at discharge	n (%)	8 (18.2)	10 (6.7)	31 (7.2)	14 (7.9)	<0.001
Death at follow-up	n (%)	22 (50.0)	15 (10.1)	56 (13.0)	51 (28.8)	<0.001
Stroke recurrence	n (%)	3 (6.8)	14 (9.4)	45 (10.5)	39 (22.0)	<0.001
Stroke recurrence (Event rate) *		21.7 (7.0– 67.3)	29.3 (17.3–49.5)	29.5 (22.0–39.5)	50.1 (36.6–68.6)	

* (per 1000 patient years).

Table 13: *Prevalence of PES and degree of their overlap stratified by cluster.*

PES sources		**Cluster 1** (n=44)	**Cluster 2** (n=149)	**Cluster 3** (n=430)	**Cluster 4** (n=177)	***p*-value**
Number of PES sources	Median (IQR)	2 (2–3)	2(1–3)	2(1–3)	2(2–3)	<0.001
Number with 2 PES sources	n (%)	20 (45.5)	42 (28.2)	134 (31.2)	81 (45.8)	<0.001
Number with ≥3 PES sources	n (%)	17 (38.6)	43 (28.7)	119 (27.7)	69 (39.0)	<0.001
Atrial fibrillation	n (%)	8 (18.2)	13 (8.7)	10 (9.3)	59 (33.3)	<0.001
Atrial cardiopathy	n (%)	32 (72.7)	48 (32.2)	103 (24.0)	177 (100)	<0.001
Arterial disease	n (%)	9 (20.5)	68 (45.6)	248 (57.7)	63 (35.6)	<0.001
Left ventricular disease	n (%)	44 (100)	77 (51.7)	223 (51.9)	91 (51.4)	<0.001
Cardiac valvular disease	n (%)	6 (13.6)	13 (8.7)	41 (9.5)	9 (5.1)	0.198
PFO	n (%)	1 (2.3)	58 (38.9)	101 (23.5)	10 (5.7)	<0.001
Cancer	n (%)	2 (4.6)	13 (8.7)	50 (11.6)	9 (5.1)	0.051

Using multivariable logistic regression models, we determined the association between each PES and cluster membership. The adjusted odds ratios and 95% CIs for each cluster are presented in **Table 14.** Left ventricular disease was perfectly associated with cluster 1 membership. PFO was significantly associated with increased likelihood of cluster 2 membership (adjusted odds-ratio 2.69, 95% CI 1.64-4.41). Arterial disease was significantly associated with increased likelihood of cluster 3 membership (adjusted odds-ratio 2.21, 95% CI 1.43-3.13). Atrial cardiopathy was perfectly associated with cluster 4 membership

Table 14: *Association and effect size between each PES and cluster membership*

	Odds ratio	95% CI	Association
Cluster 1			
Left ventricular disease	Perfectly associated with cluster*		Positive association
Arterial disease	0.22	0.09 – 0.53	Negative association
Atrial cardiopathy	1.82	0.81 – 4.05	No association
Cardiac valvular disease	1.35	0.48 – 3.79	No association
Atrial fibrillation	0.85	0.33 – 2.18	No association
Cancer	0.53	0.11 – 2.48	No association
PFO	0.24	0.03 – 1.95	No association
Cluster 2			
PFO	2.69	1.64 – 4.41	Positive association
Atrial fibrillation	0.65	0.34 – 1.28	No association
Cardiac valvular disease	1.49	0.76 – 2.94	No association
Left ventricular disease	1.17	0.76 – 1.81	No association
Arterial disease	1.16	0.72 – 1.84	No association
Cancer	1.14	0.59 – 2.23	No association
Atrial cardiopathy	0.67	0.43 – 1.03	No association
Cluster 3			
Arterial disease	2.12	1.43 – 3.13	Positive association
Atrial cardiomyopathy	0.14	0.10 – 0.20	Negative association
Cancer	1.63	0.90 – 2.96	No association
Cardiac valvular disease	1.62	0.91 – 2.90	No association
Atrial fibrillation	0.88	0.53 – 1.46	No association
Left ventricular disease	0.84	0.58 – 1.21	No association
PFO	0.69	0.44 – 1.11	No association
Cluster 4			
Atrial cardiopathy	Perfectly associated with cluster*		Positive association
Left ventricular disease	0.38	0.23 – 0.63	Negative association
Cardiac valvular disease	0.32	0.14 – 0.72	Negative association
Atrial fibrillation	1.46	0.83 – 2.55	No association
Arterial disease	0.63	0.37 – 1.08	No association
Cancer	0.47	0.20 – 1.11	No association
PFO	0.47	0.20 – 1.13	No association

*The adjusted odds ratios and 95% CIs for each cluster are ranked by significance and effect size. The regression model has been adjusted for sex, age, hypertension, dyslipidemia, diabetes mellitus, smoking, coronary artery disease, and National Institute of Health Stroke Scale score at admission . * 100% of individuals within the cluster had the condition*

7.2 New incident atrial fibrillation during follow-up

The total of 884 consecutive ESUS patient enrolled in our dataset was followed for a median of 23.7 months, corresponding to an overall follow-up period of 2,899 patient-years. Among them, during the follow-up period, 133 patients (15%) had new incident AF.

Among the 839 patients, included in the main analysis (development of a multivariate model and a score for AF prediction), 125 patients (14.9%) had new incident AF during follow up. The most frequent diagnoses were made during a routine 12-lead ECG (41.6%), by ECG/telemetry during hospitalization for a stroke recurrence (24.0%) and during a repeat Holter ECG (14.4%), with the remaining cases being diagnosed during symptomatic episodes (e.g. palpitations) or by ECG/telemetry during hospitalization for acute myocardial infarction or other non-stroke causes or during prolonged cardiac monitoring.

Comparing baseline characteristics of patients with and without new incident AF during the follow up period (summarized in **table 15**), patients who had new incident AF were older and had higher prevalence of arterial hypertension, diabetes mellitus, and coronary artery disease.

Table 15: *Baseline characteristics of patients with and without new incident AF in the overall population*

Variable	No new incident AF during follow-up (N = 714)	New incident AF during follow-up (N = 125)	Odds-ratio	95% CI
Age (years)	66.0 (23.2)	74.0 (13.7)	1.04	1.02 - 1.06
Female sex	303 / 714 (42.4%)	59 / 125 (47.2%)	1.21	0.83 - 1.77
Arterial hypertension	420 / 714 (58.8%)	102 / 125 (81.6%)	3.10	1.96 - 5.11
Dyslipidemia	479 / 714 (67.1%)	74 / 125 (59.2%)	0.71	0.48 - 1.05
Diabetes mellitus	122 / 714 (17.1%)	33 / 125 (26.4%)	1.74	1.11 - 2.69
Current smoking	283 / 714 (39.6%)	41 / 125 (32.8%)	0.74	0.49 - 1.11
Coronary artery disease	98 / 713 (13.7%)	29 / 124 (23.4%)	1.92	1.19 - 3.03
Peripheral artery disease	18 / 714 (2.5%)	6 / 125 (4.8%)	1.95	0.70 - 4.76
Heart failure	36 / 714 (5.0%)	9 / 125 (7.2%)	1.46	0.65 - 2.99
Previous stroke or TIA	119 / 714 (16.7%)	23 / 125 (18.4%)	1.13	0.67 - 1.82
Cardiac valvular disease	4 / 714 (0.6%)	1 / 125 (0.8%)	1.43	0.07 - 9.78
Cancer	66 / 714 (9.2%)	13 / 125 (10.4%)	1.14	0.58 - 2.07
Previous myocardial infarction	88 / 714 (12.3%)	18 / 125 (14.4%)	1.20	0.67 - 2.03
NIHSS score at admission	6.0 (10.0)	5.0 (7.0)	0.98	0.95 - 1.01

7.2.1 Stroke severity and new incident AF

New incident AF was detected in 13.5% of patients with severe ESUS (admission NIHSS ≥ 6) and 17% among patients with mild ESUS (admission NIHSS < 6) (unadjusted OR: 0.76, 95%CI: 0.51–1.13) **(table 16).** In the multivariable regression analysis, the likelihood of new incident AF was similar between severe ESUS and mild ESUS (adjusted OR: 0.67, 95%CI: 0.44–1.03, p ¼ 0.06). It should be noted that for the association between ESUS severity and occurrence of new incident AF, logistic regression was used.

***Table 16**: Outcomes according to ESUS severity*

Outcomes	n (annualized rate %)			
	Severe ESUS (n=414)	Mild ESUS (n=358)	Unadjusted HR (95% CI)	Adjusted HR (95% CI)
Ischemic stroke	49 (3.3)	49 (3.4)	0.98 (0.66–1.46)	1.09 (0.73–1.62)
Death	79 (5.4)	53 (3.7)	1.46 (1.03–2.06)	1.51 (1.05–2.16)
New incident AF	56 (13.5)[a]	61 (17.0)[a]	0.76 (0.51–1.13)[b]	0.67 (0.44–1.03)[b]

[a] Percent of total group count.

[b] Odds Ratio (OR) (95%CI).

7.2.2 LAD and new incident AF

From the 676 patients with available information about left atrial diameter, a diagnosis of new incident AF was made in 115 patients (17.0%). Their mean follow-up was 3.6 years corresponding to 2437 patient-years.

Comparing the prognostic performance of different LAD thresholds for the diagnosis of new incident AF, LAD threshold of 40 mm had the best prognostic performance as it yielded the highest Youden's J-statistic of 0.35 with a sensitivity of 0.69, specificity of 0.66, positive prognostic value of 0.27 and negative prognostic value of 0.92 for the prediction of new incident AF. The sensitivity, specificity, positive prognostic value, negative prognostic value and Youden's J-statistic of different LAD thresholds to predict new incident AF is summarized in **Table 16.**

***Table 17**: Prognostic performance of different LAD thresholds for the diagnosis of new incident AF*

LAD (mm)	Proportion of patients above this threshold	Proportion of patients detected with AF above this threshold	Sensitivity	Specificity	Positive predictive value	Negative predictive value	Youden's J-statistic
>37	56,4	22,8	0,85	0,49	0,23	0,95	0,34
>38	50,7	23,9	0,79	0,55	0,24	0,94	0,34
>39	46,0	24,8	0,74	0,60	0,25	0,93	0,34
>40	39,1	26,5	0,69	0,66	0,27	0,92	0,35
>41	35,2	26,9	0,63	0,70	0,27	0,91	0,32
>42	28,3	25,1	0,47	0,75	0,25	0,89	0,22
>43	22,5	23,0	0,34	0,80	0,23	0,87	0,14
>44	17,6	22,7	0,26	0,84	0,23	0,87	0,10
>45	13,0	23,9	0,21	0,88	0,24	0,86	0,09
>46	10,1	25,0	0,17	0,91	0,25	0,86	0,08
>47	9,0	26,2	0,16	0,92	0,26	0,86	0,08
>48	7,1	25,0	0,12	0,94	0,25	0,86	0,05
>49	6,2	19,0	0,08	0,94	0,19	0,85	0,02
>50	3,4	26,1	0,06	0,97	0,26	0,85	0,03
>51	2,5	29,4	0,05	0,98	0,29	0,85	0,03
>52	1,6	45,5	0,05	0,99	0,45	0,85	0,04
>53	1,5	50,0	0,05	0,99	0,50	0,85	0,04
>54	1,0	57,1	0,04	0,99	0,57	0,85	0,03

The rate of new incident AF was 9.1% in patients with LAD≤40mm (diagnosed in 37 patients after a mean of 3.3 years), and 28.9% in patients with LAD > 40 mm (diagnosed in 78 patients after a mean of 4.0 years). In the multivariate regression analysis, LAD >40 mm was associated with higher probability for new incident AF compared to LAD ≤40 mm (HR: 1.92, 95%CI: 1.24–2.97, p = 0.004). In the secondary analysis including LAD as a continuous covariate, LAD was a significant independent predictor of new incident AF (HR: 1.05, 95%CI: 1.01–1.08 for each mm increase of LAD, p = 0.006). In Kaplan–Meier analysis, the 10-year cumulative probability of new incident AF was higher in patients with LAD >40 mm compared to LAD ≤40 mm (53.5% and 22.4% respectively, log-rank test 28.2, p < 0.001) **(Figure. 6)**

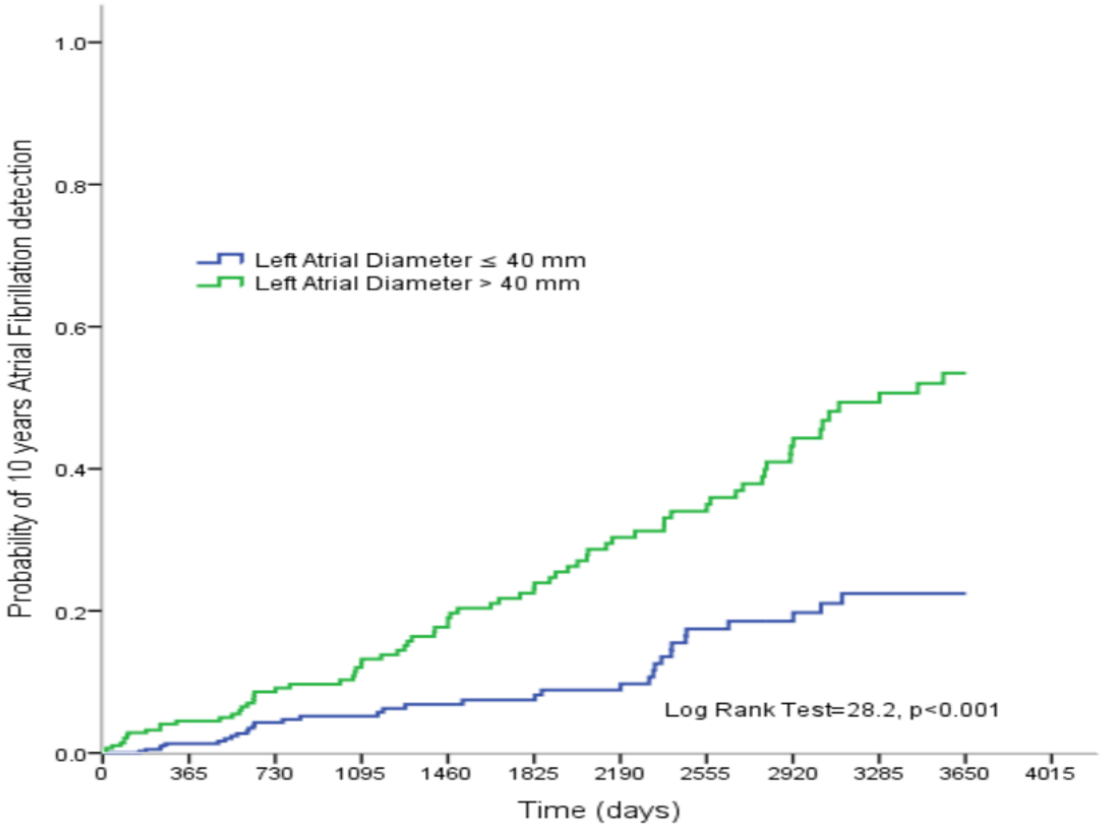


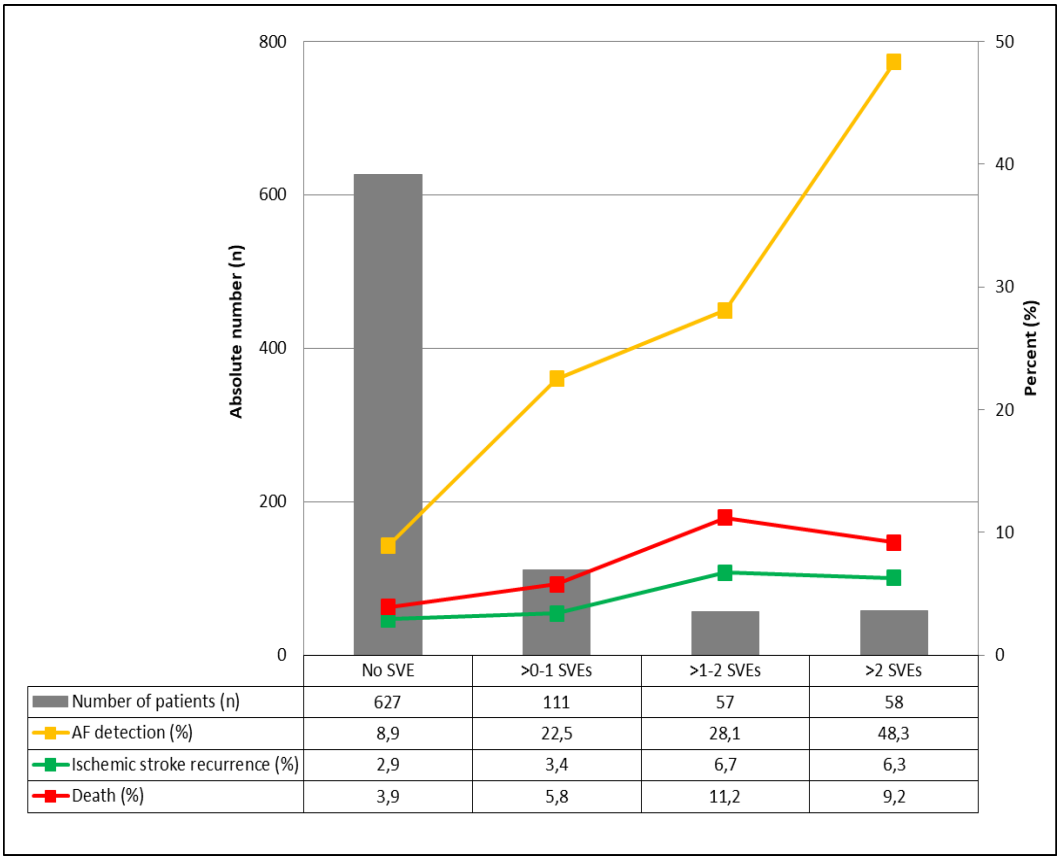
Figure 6: *10-year cumulative probability of new incident AF by LAD threshold of 40 mm*

7.2.3 SVE and new incident AF

Among the 853 patients with ECG available for evaluation in their medical archives, AF was detected in 125 patients (14.7%) during follow- up. Their mean follow-up was 3.4 years corresponding to a total of 2857 patient-years (median of 2 years; interquartile range: .8-5.8 years) and 14.5% of patients were lost to follow up.

Comparing AF detection between the different SVE groups (as described in paragraph 7.1.3.2), the rate of AF detection was 8.9% in patients with no SVE, 22.5% in patients with greater than 0-1 SVEs, 28.1% in patients with greater than 1-2 SVEs and

48.3% in patients with greater than 2 SVEs **(Figure 7)**. The negative prognostic value of the presence of any SVE for the prediction of new AF was 91.4% (95%CI: 88.8%-93.3%). In the multivariate regression analysis, SVEs were associated with the probability of AF detection **(Figure 8).** The 10-year cumulative probabilities of AF detection were 30.7% (95%CI: 22.7-38.7), 54.6% (95%CI:37.4-71.8), 59.2% (95%CI: 38.6%-79.8%) and 81% (95%CI: 63.9%-98.1%) in patients with no SVE, greater than 0-1, greater than 1-2 and greater than 2 SVEs respectively **(Figure 9)**.



	No SVE	>0-1 SVEs	>1-2 SVEs	>2 SVEs
Number of patients (n)	627	111	57	58
AF detection (%)	8,9	22,5	28,1	48,3
Ischemic stroke recurrence (%)	2,9	3,4	6,7	6,3
Death (%)	3,9	5,8	11,2	9,2

Figure 7: *Frequency distribution, overall rate of AF detection and annualized event rates of ischemic stroke recurrence and death by SVE group*

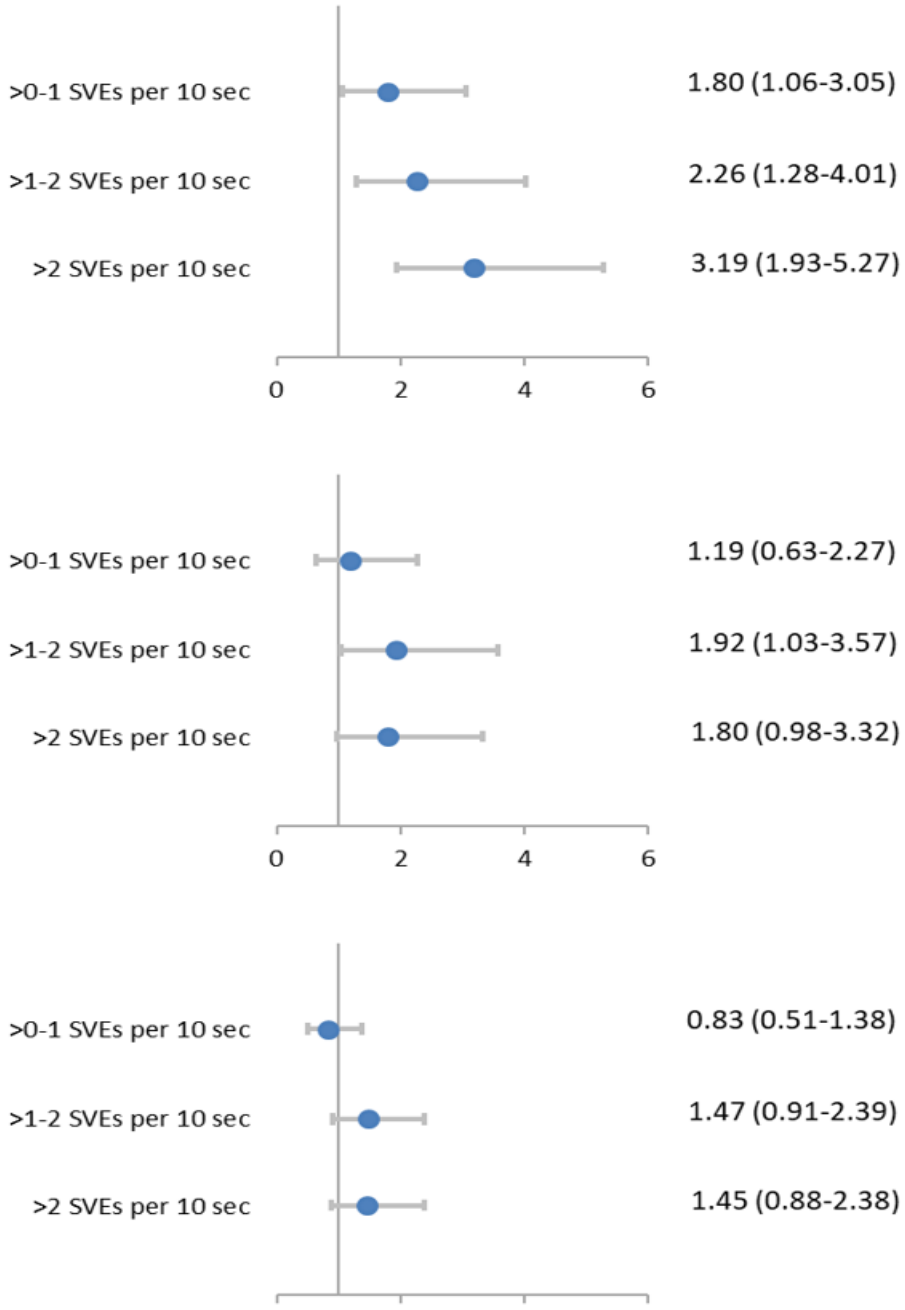


***Figure 8**: Multivariate regression analyses of the association between SVEs groups and AF detection, ischemic stroke recurrence and death*

AF detection *(upper panel)*, ischemic stroke recurrence *(middle panel)* and death *(lower panel)*. Comparisons are made to patients with no SVE.

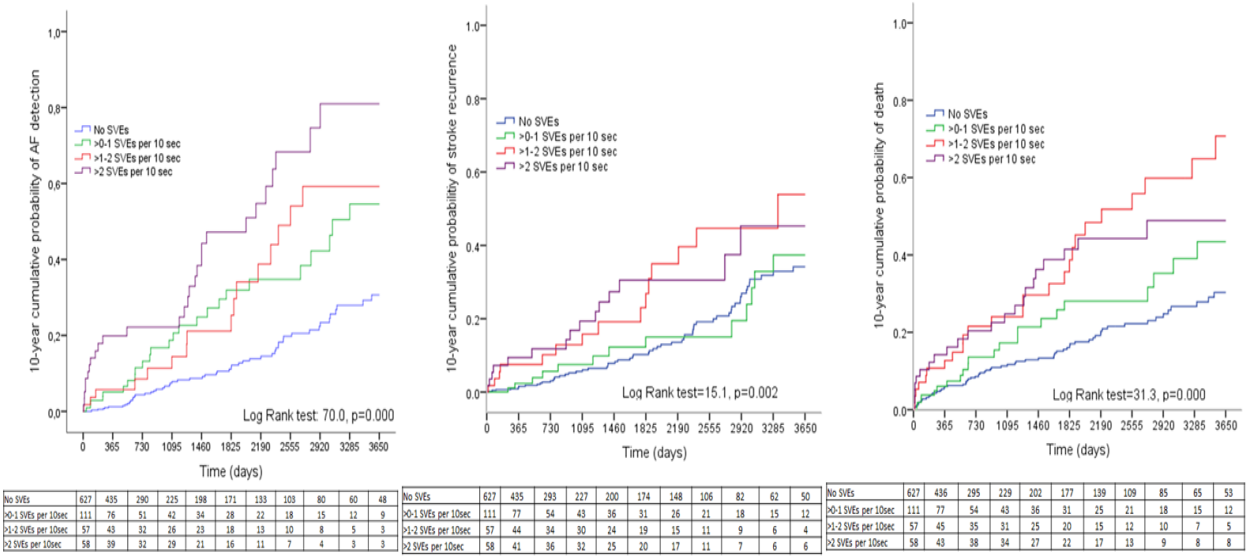


Figure 9: *10-year cumulative probability of AF detection, ischemic stroke recurrence and death by SVE group*

AF detection (left panel), ischemic stroke recurrence (middle panel), death (right panel)

7.2.4 Non- stenotic carotid plaques and new incident AF

Among 777 patients with available information about the presence or absence of ipsilateral nonstenotic carotid plaques, AF was detected in 112 (14.4%) patients during follow-up at a median of 23 months (interquartile range 9–64 months).

The overall rate of AF detection was 8.5% in patients with ipsilateral nonstenotic carotid plaques (corresponding to 2.9% per 100 patient-years), compared to 19.0% in patients without (corresponding to 5.0% per 100 patient-years) (unadjusted HR 0.56, 95% CI 0.37–0.84). In the multivariate regression analysis, the presence of ipsilateral nonstenotic carotid plaques was associated with lower probability for AF detection (adjusted HR 0.57, 95% CI 0.34–0.96, p = 0.03). In Kaplan-Meier analysis, the 10- year cumulative probability of AF detection was lower in patients with ipsilateral nonstenotic

carotid plaques compared to those without, 34.5% (95% CI 21.8–47.2) and 49.0% (95% CI 40.4–57.6), respectively (log-rank test 11.8, p = 0.001, **figure 10)**

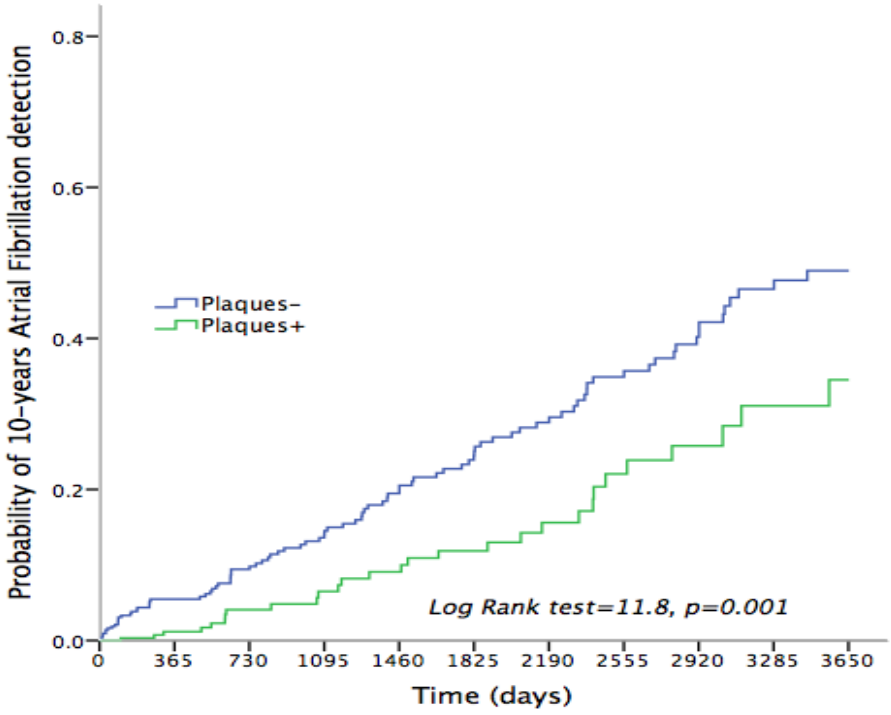


Figure 10: *Ten-year cumulative probability of AF detection in patients with and without nonstenotic carotid plaques*

7.2.5 PFO and new incident AF

Among the 455 patients, with known PFO status, 11 (2%) had missing follow-up data. The remaining 444 patients with available follow-up had a median follow-up of 1.7 years. During follow-up, 34 patients (7.6%) had new incident AF. None of the new incident AF was observed in the post-procedural phase after PFO closure: in the 4 patients with

previous PFO closure and new incident AF, this was observed in a range between 6 months and 7 years after the procedure.

For the follow-up analyses, 3 groups of patients were identified: without PFO, with incidental PFO, and with pathogenic PFO (defined as RoPE score 0–6 and 7–10, respectively, according to previous results (225)). In Kaplan-Meier analysis, the 10-year cumulative probability of new incident AF was lowest in patients with likely pathogenic PFO (RoPE=7–10), intermediate in those with likely incidental PFO (RoPE=0–6), and highest in those without PFO (log-rank test= 6.28, P=0.04; **Figure 11**).

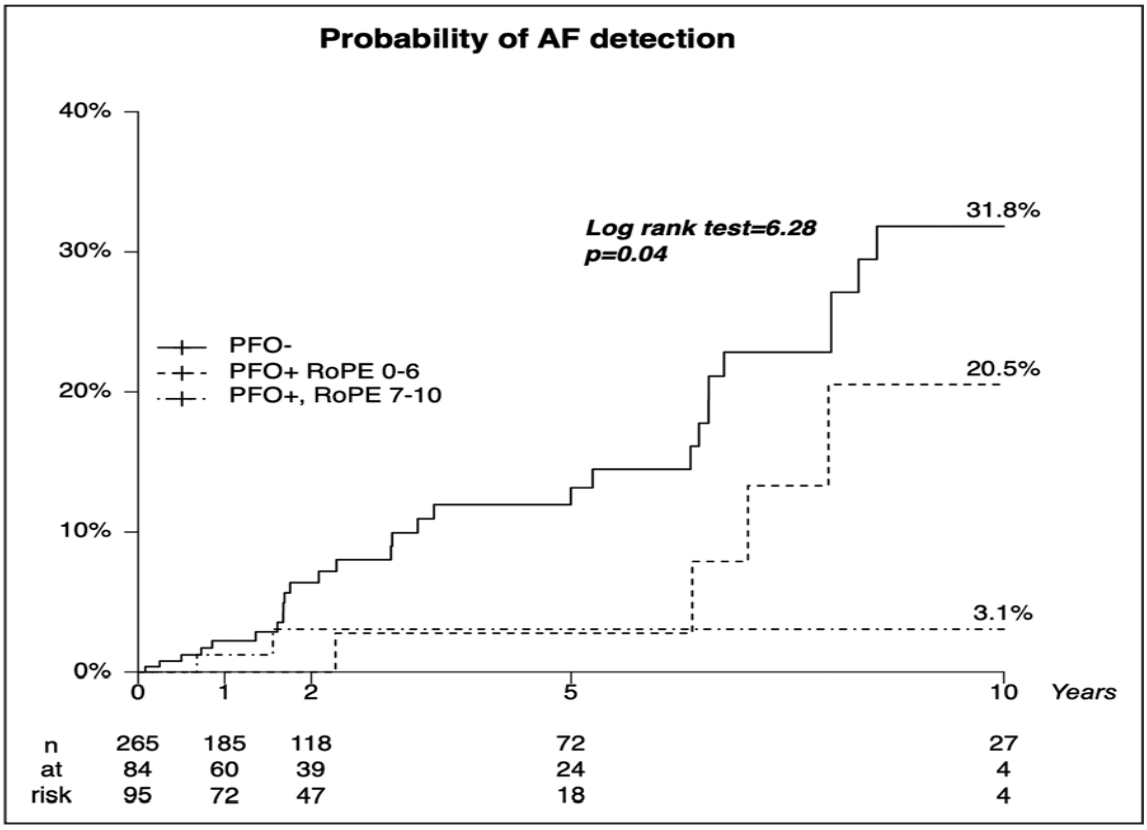


Figure 11: *Cumulative probability of new incident AF in patients without PFO, likely incidental PFO (RoPE score 0–6), and likely pathogenic PFO (RoPE score 7–10).*

7.3 Development of the multivariate model and score for AF prediction

7.3.1 Methods

7.3.1.1 Development of the multivariate model.

We performed multivariate stepwise regression with forward selection of covariates to identify independent predictors of new incident AF. The covariates that were finally included in the stepwise regression are presented in the **table 18**. Some parameters, as left atrial volume or PFO presence were excluded from the multivariate analysis due to a lot of missing data. For patients lost during follow up, survival data were censored at the last time known to be alive. Missing values were imputed using the chain equations technique. Five datasets were generated, which were subsequently analyzed separately. In each analysis, stepwise methods were implemented to select the statistically significant covariates. The results of the five analyses were appropriately combined to generate the final multivariate model. To test for collinearity between the covariates of the final multivariate model, we calculated the adjusted generalized variance inflation factor (VIF) for each covariate. Continuous covariates are summarized as median and interquartile range; nominal variables are given as count and absolute percentages. All comparisons were performed using the Cochran–Mantel–Haenszel chi-squared test. Associations are presented as odds ratios (ORs) with their corresponding 95% confidence intervals (95%CI) and the level of significance was set at 5%. Statistical analyses were performed with the R package (version 3.5.3)

***Table 18**: Covariates included in the multivariate stepwise regression for the development of the multivariate model*

Demographics
age, sex
Presenting clinical symptoms as reported by the treating stroke physician
stroke severity, aphasia, dysarthria, eye deviation, paresis, sensory symptoms, neglect, hemianopia, impaired consciousness, brainstem symptoms
Medical history
arterial hypertension, dyslipidemia, diabetes, current smoking, coronary artery disease, peripheral artery disease, heart failure, previous stroke or transient ischemic attack (TIA), obesity, cancer, alcohol consumption
Acute brain imaging findings as reported by board-certified consultant radiologists
localization, cortical/subcortical infarct, single- or multi-territorial infarct, lateralization, leukoaraiosis, brain oedema, hemorrhagic transformation, chronic infarct, ASPECTS score
Electrocardiographic parameters in all available 12-lead standard ECGs performed during hospitalization for the ESUS
atrioventricular blocks, ST segment elevation, PR interval duration, supraventricular and ventricular extrasystoles, Sokolov index
Echocardiographic parameters as reported by board-certified consultant cardiologist
left atrial diameter, left ventricular ejection fraction, aortic and mitral stenosis/regurgitation, left ventricular hypertrophy, left ventricular wall thickness, interventricular septum thickness, end-diastolic left ventricular volume
Carotid imaging parameters
presence of ipsilateral or contralateral non-stenotic plaques, i.e. causing <50% stenosis) and treatment at discharge (antiplatelet or oral anticoagulant

7.3.1.2 Development of the score from the multivariate model

Simplification of the coefficients of the linear predictor of the final multivariate model was performed to develop the proposed score. The coefficient of each independent covariate of the fitted multivariable model was used to generate an integer-based point scoring system by dividing each covariate with the smallest coefficient and then rounded to the nearest integer. The individual risk scores of all covariates were summed and a total risk score was calculated for each patient

7.3.1.3 Assessment of the accuracy of the score

To assess the accuracy of the final multivariate model and the accuracy of the developed score, we examined two indices of accuracy discrimination and calibration (226). Discrimination was defined as the degree to which the prognostic score enables the discrimination between patients with favourable and unfavorable outcome and was assessed by the calculation of the area under the receiver operating characteristic curves (area under the curves (AUCs)). Calibration was defined as the agreement between predicted and actual outcome and was assessed with the use of the Hosmer–Lemeshow goodness-of-fit test with 10 groups. We also assessed the sensitivity and the negative predictive value of the score for specific thresholds. The sensitivity for a specific score threshold was defined as the probability that the score of the patient is higher than this threshold if new incident AF is diagnosed during follow-up (or else, true positive rate). The negative predictive value for a specific score threshold was defined as the probability that new incident AF is not diagnosed during follow-up if the score is below this threshold.

7.3.1.4 Internal validation of the score

An internal validation tool aiming to identify how the final multivariate model compares to the score model in terms of prediction error was carried out. We followed the cross-validation (CV) approach which splits randomly the data in 10 groups of roughly equal size, implements a model in 9/10 of the data, and assesses its predictive ability in the remaining part. This process was repeated for each group separately and an average internal prediction error was evaluated. The influence of the initial split on the prediction error estimate was diminished by splitting more than once (in fact, 1000 replications of the

splitting process were performed) and calculating the CV error for each split. An accurate estimate of the standard error of the CV error was derived from the above procedure allowing formal statistical inference.

7.3.1.5 External validation of the score

In order to external validate the developed score, we assessed its performance in a previously published prospective cohort of consecutive ESUS patients with prolonged cardiac monitoring (227). The cohort included consecutive patients with ESUS admitted to the stroke unit of the Department of Neurology of the Evangelisches Klinikum Bethel between June 2013 and January 2015 (227). All patients were submitted to prolonged cardiac monitoring with the Reveal XT® (Medtronic Inc., Minneapolis, MN, USA) or BioMonitor® (Biotronik Co., Berlin, Germany) implantable loop recorders (ILR) (227). The developed AF-ESUS score was calculated for all ESUS patients, who were stratified based on several AF duration thresholds (>24 h; 10 h; 6 h; 1 h; 6 min and no AF). To assess the performance of the score we used a confusion matrix to calculate true-/ false-positives (TP and FP, respectively) and true-/ false-negatives (TN and FN, respectively). The sensitivity of AF-ESUS was defined as the probability that the AF-ESUS score will be >0 if AF is present [TP/(TP+FN)] and negative predictive value (NPV) was defined as the probability that AF is not present if AF-ESUS≤0 [TN/(TN+FN)] for various thresholds of AF duration.

7.3.2 Results

As already mentioned, because of missing data or unavailable follow-up, from the total of 884 registered ESUS patients, 839 were included in the main analysis for the development of the multivariate model and the score for prediction of AF. 43.1% of them were women and their median age was 67 years (interquartile range (IQR): 54–77). Among these patients, 93.1% were discharged on antiplatelet treatment. The overall follow-up period was 2999 patient-years, corresponding to a median follow-up of 24.3 months (IQR: 10.0–70.0). The baseline characteristics of these patients are summarized in the **table 15**.

7.3.2.1 Multivariate model and score for AF prediction

The final multivariate model included eight covariates **(Table 19).** The adjusted generalized VIF of the covariates in the final multivariate model ranged between 1.03 and 1.11, with a mean VIF of 1.06, showing that collinearity is not an issue in our analysis, since the estimated VIFs are well below the threshold of 10 for all variables include in our score. The type of antithrombotic treatment (i.e. anticoagulant or antiplatelet) was not associated with any of the outcomes. The scoring system which was developed by the final multivariate model and the points assigned to each covariate are also presented in Table 4. The proposed score assigns 3 points for age ≥ 60 years, 2 points for arterial hypertension, -1 point for left ventricular hypertrophy reported at echocardiography, 2 points for left atrial diameter >40 mm, -3 points for left ventricular ejection fraction <35%, 1 point for the presence of any supraventricular extrasystole recorded during all available 12-lead standard ECGs performed during hospitalization for the ESUS, -2 points for subcortical infarct and -3 points for non-stenotic carotid plaque (either ipsilateral or contralateral to the ischemic

territory). The median score was 2 (IQR: 0–4) in the overall population, 5 (IQR: 3–6) in patients with new incident AF during follow-up, and 1 (IQR: 1 to 4) in patients without new incident AF during follow-up ($p < 0.01$). In the overall cohort, 42.3% of patients had a score of ≤0. **Figure 12** presents the number of patients (left vertical axis) and the predicted probability of new incident AF (right vertical axis) per score value. The rate of new incident AF during follow-up was 26.9% among patients with a score of >0 and 1.97% among patients with a score of ≤0 (relative risk: 13.7, 95%CI: 5.9–31.5). The sensitivity and the negative predictive value of a score of ≤0 for new incident AF during follow-up were 94.9% (95%CI: 89.3–98.1%) and 98.0% (95%CI: 95.8–99.3%) respectively.

***Table 19**: Covariates included in the final multivariate model, odds-ratios, log (odds-ratios) and points assigned to each covariate for the score calculation*

Covariate	OR (95%CI)	p-value	logOR (95%CI)	Points assigned for score calculation
Age				
• **60 to 70 years**	5.55 (2.62-11.78)ǂ	<0.001	1.71 (0.96-2.47)	**3**
• **>70 to 80 years**	4.95 (2.35-10.46)ǂ	<0.001	1.60 (0.85-2.35)	**3**
• **>80 years**	5.26 (2.28-12.16)ǂ	<0.001	1.66 (0.82-2.50)	**3**
Arterial hypertension	2.47 (1.40-4.37)	<0.01	0.90 (0.33-1.47)	**2**
Left ventricular hypertrophy#	0.52 (0.31-0.87)	0.01	-0.65 (-1.16 to -0.14)	**-1**
Left atrial diameter >40mm	2.59 (1.59-4.20)	<0.001	0.95 (0.46-1.43)	**2**
Left ventricular ejection fraction <35%	0.26 (0.10-0.71)	0.001	-1.34 (-2.33 to -0.34)	**-3**
Any supraventricular extrasystole†	1.89 (1.18-3.05)	<0.01	0.64 (0.16-1.11)	**1**
Subcortical infarct	0.44 (0.27-0.72)	0.001	-0.81 (-1.30 to -0.33)	**-2**
Non-stenotic carotid plaque^	0.24 (0.15-0.40)	<0.001	-1.42 (-1.93 to -0.91)	**-3**

ǂ compared to patients <60 years
reported at echocardiography.
† recorded during all standard 12-lead ECGs performed during hospitalization for the ESUS
^ ipsilateral or contralateral to the ischemic territory

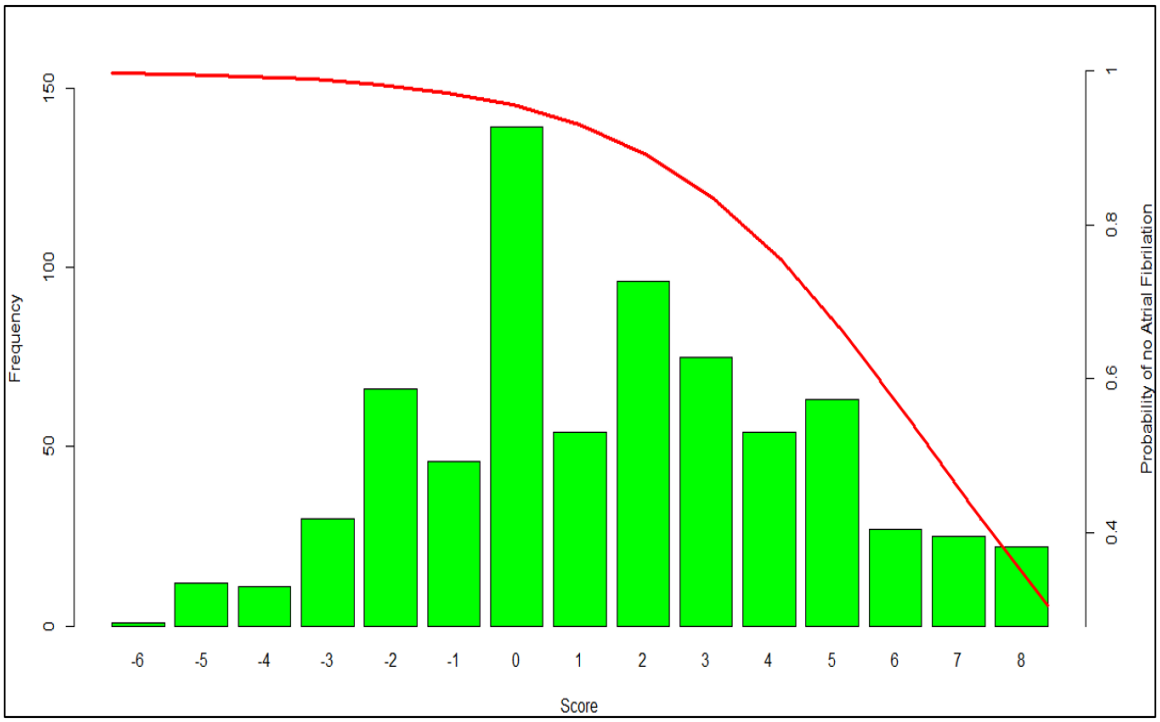


Figure 12: *Number of patients and predicted probability of new incident AF per score value*

bars and left vertical axis → *Number of patients*, continuous line and right vertical axis → *predicted probability of new incident AF*, horizontal axis → *score value.*

7.3.2.2 Accuracy of the score and internal validation

The AUC was 83.8% (95%CI: 79.9–86.9%) for the final multivariate model and 84.8% (95%CI: 79.9– 86.9%) for the score **(Figure 13)**. The Hosmer– Lemeshow statistic was 6.55 (p = 0.59) for the final multivariate model and 4.85 (p = 0.77) for the score, showing acceptable calibration performance. The CV error for the final multivariate model is estimated at 10.3% (95%CI: 8.8–11.7%), while the corresponding metric for the score is similar (10.0%, 95%CI: 8.6–11.4%), indicating that our score could have an acceptable performance in terms of prediction error in external datasets with similar structure.

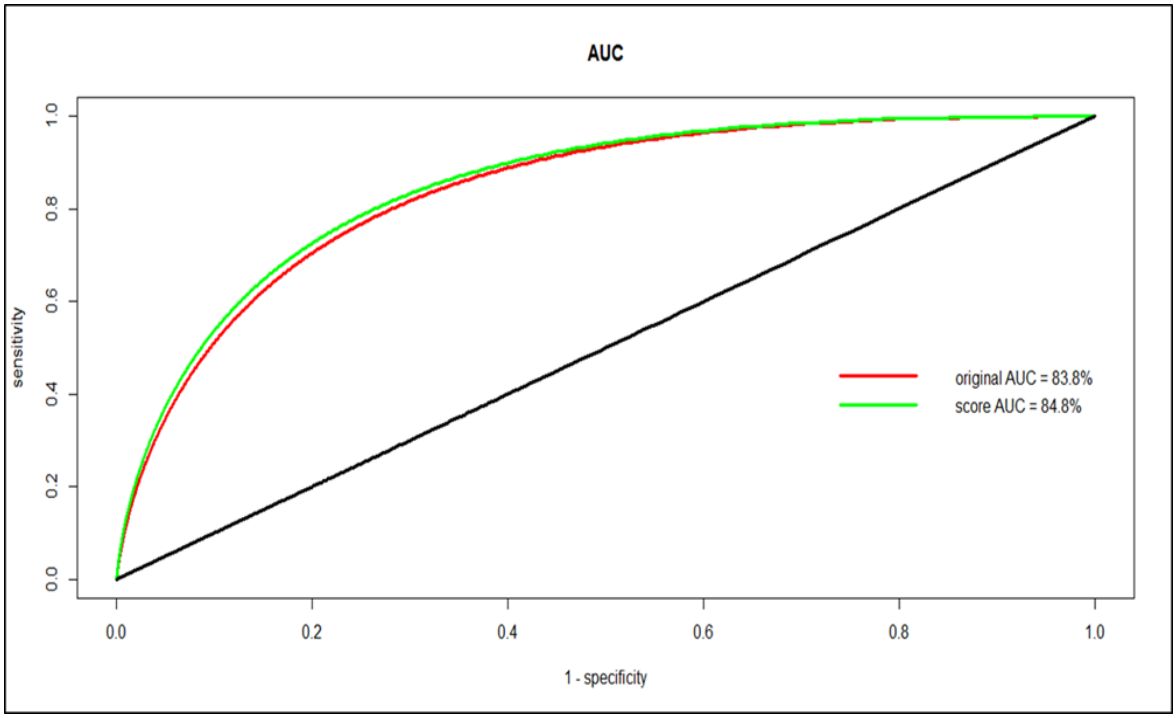


Figure 13: *Area under the receiver operating characteristic curves (AUCs) for the final multivariate model and the score*

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7.3.2.3 External validation of the score

The external validation cohort included 123 patients (40% women, median age 66 years, IQR:59-73). Among them, 31 (25.2%) had an AF-ESUS ≤0. The overall follow-up period was 373 patient-years corresponding to a median of 36 months (IQR:36-42), and AF was detected in 52 (42.3%) patients. Among patients with AF, 12 (23.1%) had AF-ESUS score ≤0. The median AF-ESUS score was 2 (IQR:0-3) in the overall population. **Table 20** presents the true- and false-positives, true- and false- negatives, sensitivities and NPV of the AF-ESUS score ≤0 for various thresholds of AF duration (>24 h; 10 h; 6 h; 1 ; 6 min). The sensitivity and NPV of the AF-ESUS threshold ≤0 was 100% for the identification of ESUS patients with AF episodes lasting >10 h; 80% and 83.9% respectively for AF episodes >6 h; and 76.6% and 64.5% respectively for AF episodes >6

min. In this cohort of consecutive ESUS patients who were monitored with ILR for 3 years after the index event, the threshold of AF-ESUS score ≤0 showed high sensitivity and high NPV to identify a considerable proportion (25%) of the overall cohort with low likelihood of AF.

Table 20: *Performance of the AF-ESUS score ≤0 for several thresholds of AF duration*

Threshold of AF duration	True positive	False positive	True negative	False negative	Sensitivity	Negative predictive value
>24 h	4	88	31	0	100%	100%
>10 h	8	84	31	0	100%	100%
>6 h	20	72	26	5	80%	83.9%
>1 h	30	62	22	9	76.9%	71%
>6 min	36	56	20	11	76.6%	64.5%

7.4 Additional analyses

7.4.1 Stroke recurrence during follow-up

As described in paragraph 6.5, stoke recurrence was one of the secondary outcomes in our study. During the median follow-up of 23.7 months, corresponding to an overall follow-up period of 2,899 patient-years, 108 patients (12.2%) had a stroke recurrence corresponding to 3.73 (95% CI 3.1–4.5) stroke recurrences per 100 patient-years.

7.4.1.1 ESUS severity and stroke recurrence

The annualized rate of stroke recurrence was 3.3% in patients with severe ESUS (admission NIHSS ≥ 6) and 3.4% in patients with mild ESUS (admission NIHSS < 6) (unadjusted HR: 0.98, 95%CI: 0.66–1.46) **(table 16).** In the multivariable regression analysis, the two groups had similar risk for stroke recurrence (adjusted HR: 1.09, 95%CI: 0.73– 1.62, p = 0.680). The 10-year cumulative probability for stroke recurrence was 38.1% (95%CI: 29.2–48.6) in severe ESUS and 36.6% (95%CI: 27.8–47.0) in mild ESUS (log-rank test: 0.01, p=0.920; **Figure 14**).

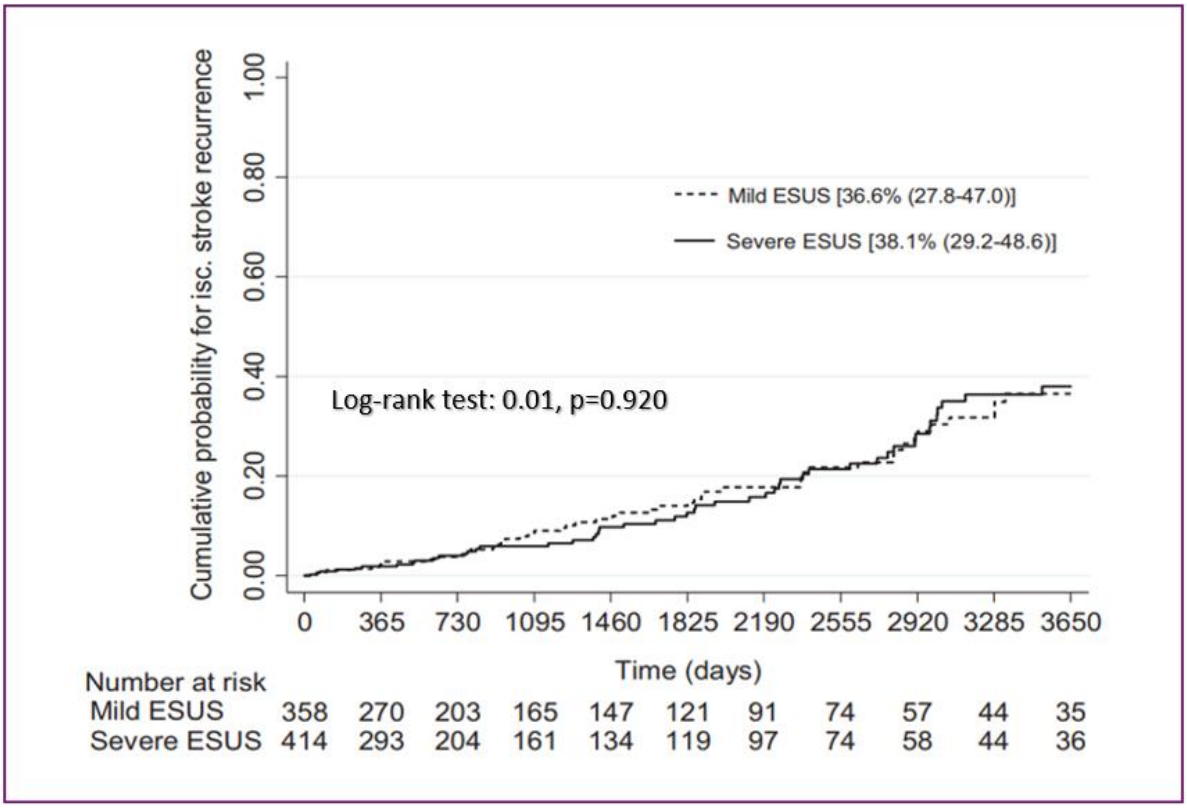


Figure 14: *Ten-year cumulative probabilities of stroke recurrence according to ESUS severity*

7.4.1.2 LAD and stroke recurrence

In the 676 patients with available information about left atrial diameter, the annualized rate of stroke recurrence was 4.0% (occurring in 98 patients at a mean interval of 3.6 years). The annualized rate of stroke recurrence was 3.7% in patients with LAD ≤40 mm (occurring in 45 patients at a mean interval of 3.4 years), and 4.9% in patients with LAD >40 (occurring in 53 patients at a mean interval of 4.0 years). In the multivariate regression analysis, LAD >40 mm was not associated with higher probability for stroke recurrence compared to LAD ≤ 40 mm (HR: 0.96, 95%CI: 0.62–1.48, p = 0.85).

7.4.1.3 SVE and Stroke Recurrence

Among the 853 patients with ECG available for evaluation in their medical archives, a recurrent ischemic stroke occurred in 103 (12.1%) patients during follow up. The annualized rate of recurrent ischemic stroke was 2.9% in patients with no SVE, 3.4% in patients with greater than 0-1 SVEs, 6.7% in patients with greater than 1-2 SVEs and 6.3% in patients with greater than 2 SVEs **(Figure 7)**. In the multivariate regression analysis, the number of SVEs was not associated with the risk of ischemic stroke recurrence **(Figure 8)**. The 10-year cumulative probabilities of ischemic stroke recurrence were 34.2% (95%CI: 26.2-42.2), 37.4% (95%CI:19.0-55.8), 53.9% (95%CI: 31.0%-76.8%) and 45.3% (95%CI: 23.9%-66.7%) in patients with no SVE, greater than 0-1, greater than 1-2 and greater than 2 SVEs respectively **(Figure 9).**

7.4.1.4 Nonstenotic carotid plaques and stroke recurrence

In the 777 patients with available information about the presence or absence of ipsilateral nonstenotic carotid plaques, followed for a mean of 3.4 years, the annualized rate of stroke recurrence was 3.8%, occurring in 101 patients. The annualized rate of stroke recurrence was 4.2% in patients with ipsilateral nonstenotic carotid plaques (occurring in a total of 42 patients) and 3.6% in patients without (occurring in a total of 59 patients) (unadjusted HR 1.27, 95% CI 0.85–1.89). In Kaplan-Meier analysis, the 10-year cumulative probability for stroke recurrence was not statistically different between patients with ipsilateral nonstenotic carotid plaques and those without (35.9%, 95% CI 27.3–44.5; 45.9%, 95% CI 33.6–58.2, respectively, log-rank test 0.947, p = 0.331) (**figure 15).**

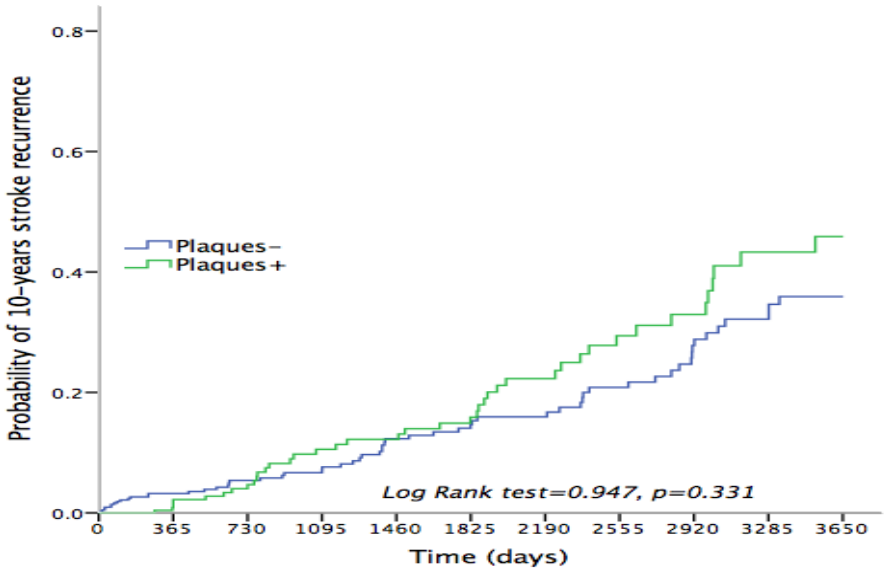


Figure 15: *10-year cumulative probability of stroke recurrence in patients with and without non-stenotic carotid plaques ipsilateral to the index stroke.*

7.4.1.5 PFO and stroke recurrence

Among the 444 patients with known PFO status and available follow-up, 37 patients (8.1%) had at least one stroke recurrence. The recurrence rate in patients with closure was 4.4% over a median of 1.8 (1–2.8) years and without closure was 7.8% over a median of 1.9 (0.8–6.2) years. In Kaplan-Meyer analysis, the estimated 10-year stroke recurrence rates were 42% and 36% respectively (log-rank test=0.2, P=0.7). The 10-year cumulative probability of stroke recurrence was lowest in likely pathogenic PFO patients (1.7%), intermediate in patients without PFO (33.8%), and highest in incidental PFO (53.0%) (**Figure 16**). This difference nearly reached statistical significance (log-rank test=5.28, P=0.07).

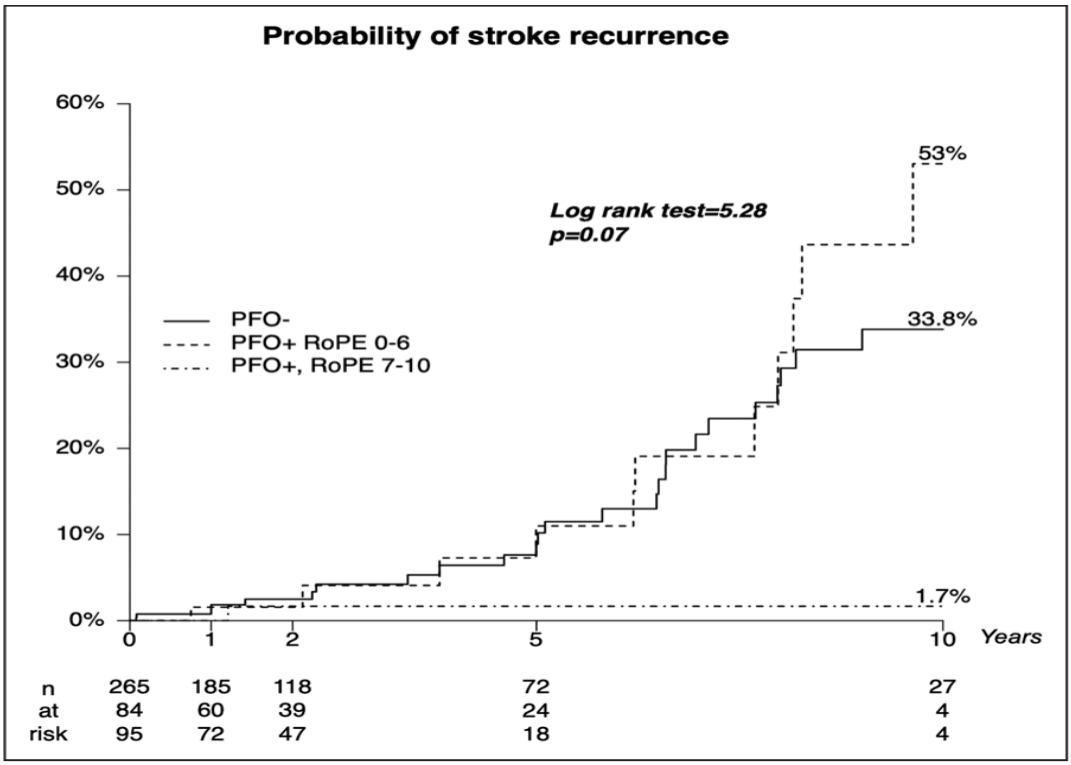


Figure 16: *Cumulative probability of stroke recurrence in patients without PFO, likely incidental PFO (RoPE score 0–6), and likely pathogenic PFO (RoPE score 7–10).*

7.4.1.6 Potential embolic sources (PES) and stroke recurrence

During a median follow-up of 3.7 years, stroke recurrence occurred in 101 (12.6%) of the 839 patients with available information, corresponding to 23.3 recurrences per 100 patient-years. The rates of stroke recurrence according to PES are presented in the **Table 11**. In multivariate analysis, the risk of stroke recurrence was higher in the AF group compared with other PES groups (Figure 11). In Kaplan-Meier analysis, the cumulative probabilities of stroke recurrence were borderline statistically different across PES groups (likelihood ratio test, 14.12; P=0.05) (Figure 12) because of the higher cumulative probability of recurrence in the AF group relatively to the other PES groups. The rates of stroke recurrence for patients with 0 to 1, 2, or ≥3 PES were 20.8, 27.6, and 21.9 per patient-years, respectively. In multivariate analysis, the risk of stroke recurrence was not statistically different between patients with 0 to 1, 2, or ≥3 PES **(Figure 17).** In Kaplan-Meier analysis, the cumulative probability of stroke recurrence was not statistically different between patients with 0 to 1, 2, or ≥3 PES (likelihood ratio test, 2.54; P=0.28) **(Figure 18)**.

7.4.1.7 Risk of stroke recurrence across clusters

During a mean follow-up duration of 3.7 years (SD 3.7), there were 101 recurrent strokes among clusters, corresponding to an overall rate of 34.6 per 1000 person-years (95% CI 28.4–42.0). The risk of stroke recurrence was not different across clusters in adjusted models (**Table 21** and **Figure 19**).

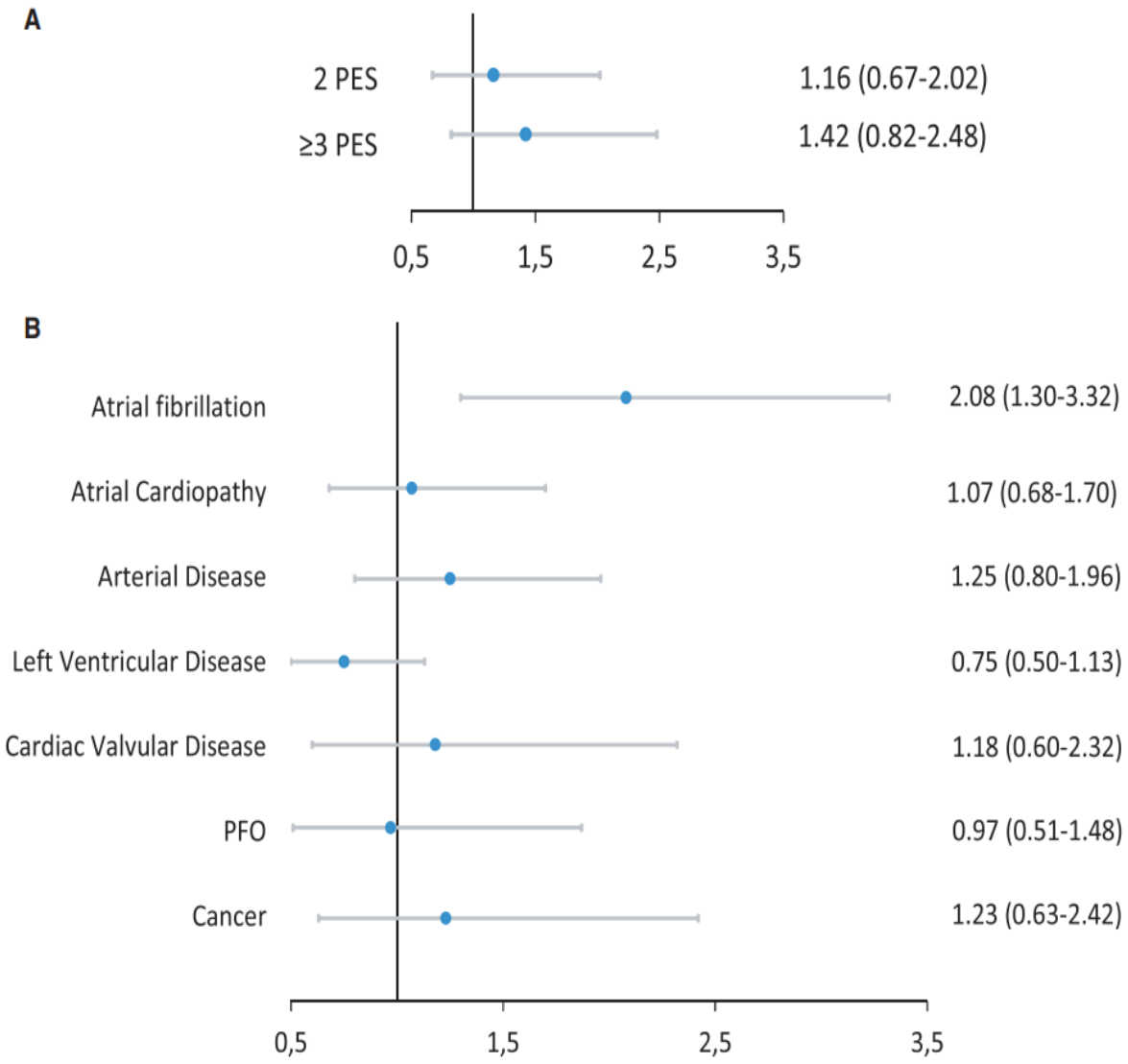


Figure 17: *Multivariable regression analysis of the association between PES and stroke recurrence*

A: *number of PES per patient and stroke recurrence (the comparisons are made to patients with 0 to 1 PES)* ***B:*** *presence of each PES and stroke recurrence (for each PES, the comparison is made to patients without the specific PES).*

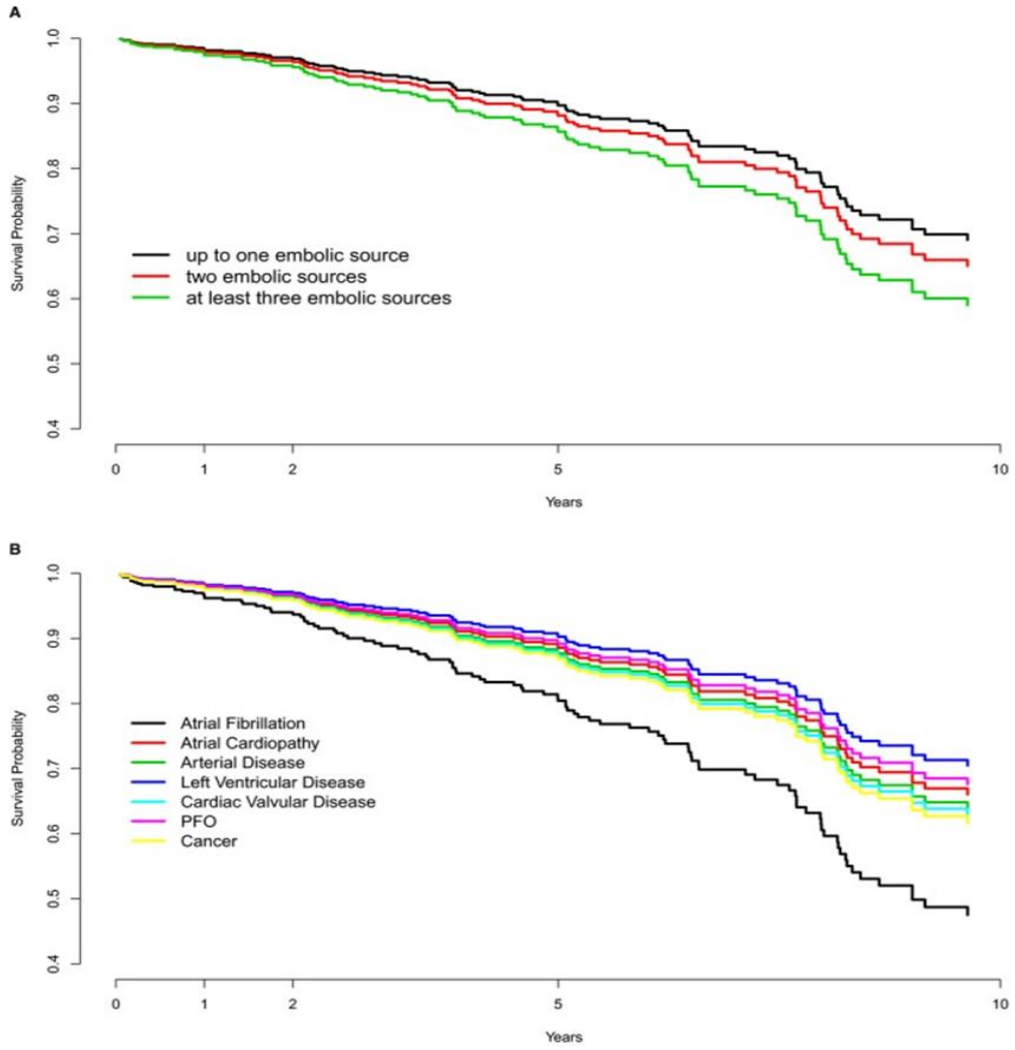


***Figure 18**: Ten-year survival estimates of stroke recurrence according to: (A) the number of PES per patient and (B) each potential embolic source*

***Table 21:** Multivariable regression analysis of the association between the phenotype clusters and stroke recurrence*

	Hazard ratio	**95% CI**
Cluster 1	Reference	Reference
Cluster 2	1.57	0.43 – 5.72
Cluster 3	1.41	0.42 – 4.72
Cluster 4	2.14	0.65 – 7.07

Cox proportional hazards regression analysis. The association has been adjusted for sex, age, hypertension, dyslipidemia, diabetes mellitus, smoking, coronary artery disease, and NIHSS at admission. The cluster with the lowest event rate for stroke recurrence was used as the reference group

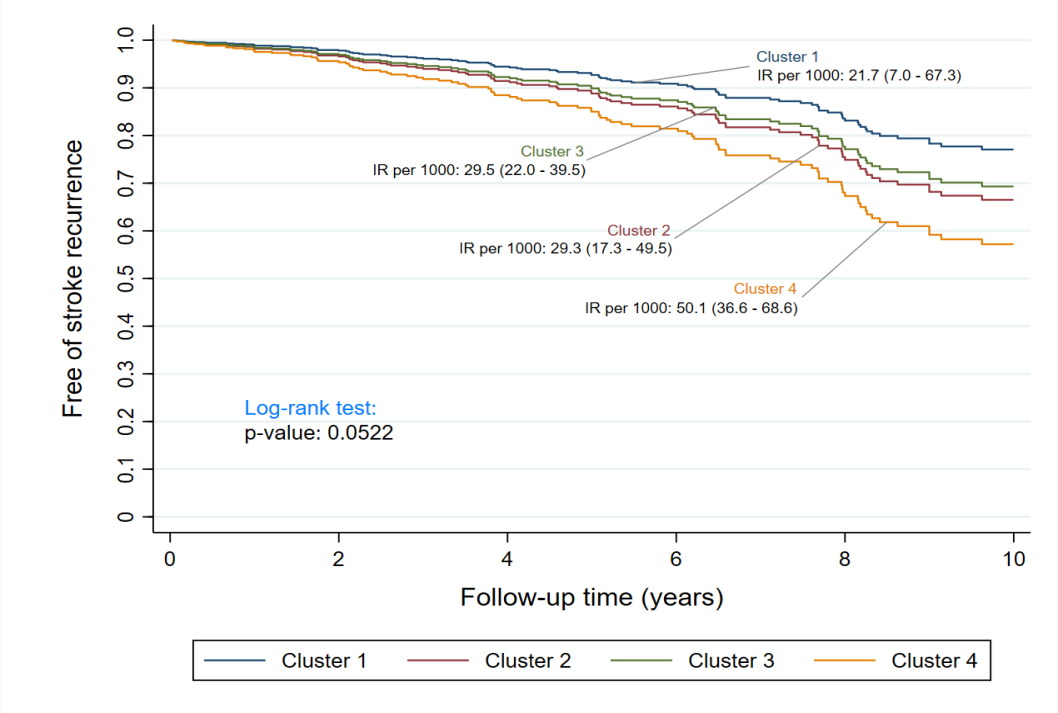


Figure 19: *Ten-year survival estimates of stroke recurrence, according to the assigned phenotype clusters*

7.4.1.8 Score for prediction of stroke recurrence

7.4.1.8.1 Methods

For the derivation of the score for prediction of stroke recurrence we used all available data from our ESUS dataset (derivation cohort) while for the external validation of the score, we used data of all consecutive ESUS patients registered in 8 other stroke registries [from Buenos Aires (Argentina), San José (Costa Rica), Helsinki (Finland), Perugia (Italy), Mexico city (Mexico), Barcelona and Madrid (Spain)] (228–234) (validation cohort).

Statistical analysis

We performed multivariable Cox regression analysis to identify predictors at index event that were independently associated with stroke recurrence. To avoid bias in building the multivariable model, we prespecified a set of biologically plausible predictors and subsequently performed a bootstrap resampling procedure (235). We conducted 1,000 forward and backward repetitions and retained variables that were selected in more than 80% of these loops. The following covariates were included in the analysis: age, sex, hypertension, smoking, diabetes mellitus, coronary artery disease, heart failure, detection of AF during follow up, left atrium size, body mass index, stroke severity on admission assessed by the National Institutes of Health Stroke Scale, leukoaraiosis, multi-territory infarct, peripheral artery disease, aortic stenosis, white blood cell count, anti-platelet and anticoagulation therapy at discharge. Leukoaraiosis was defined as patchy or diffuse areas of hypodensity on CT or hyperintensity on T2-weighted MRI in periventricular or subcortical regions, or in the pons (236), and was diagnosed by board-certified consultant radiologists. Associations are presented as coefficients and hazard ratios (HR) with their corresponding 95% confidence intervals (95% CI). To test for collinearity between the covariates of the final multivariable model, we calculated the tolerance and variance inflation factor (VIF) of each covariate.

The coefficient of each independent covariate of the fitted multivariable model was used to generate an integer-based point scoring system by dividing each covariate with the smallest coefficient and then rounded to the nearest integer; the overall score was calculated as the sum of the covariates' weighted scores. Individual risk score of all predictors were summed and a total risk score was assigned to each patient.

We compared the risk of stroke recurrence between different risk groups and used the Kaplan–Meier product limit method to estimate the cumulative probability of stroke recurrence in different risk groups. Differences in Kaplan–Meier curves were evaluated with the log-rank test.

Once again, to validate the score, we examined two indices: discrimination and calibration. Discrimination, i.e., the degree to which the prognostic score enables the discrimination between patients with and without stroke recurrence, was assessed by calculation of the area under the receiver operating characteristic curves (AUCs) in the derivation and validation cohorts. Calibration, i.e., the agreement between predicted and actual outcome, was assessed in the derivation and validation cohorts with the use of the Hosmer-Lemeshow goodness-of-fit test with 10 groups and was graphically depicted in calibration plots.

Statistical analyses were performed with STATA statistical software, version 13.1 (StataCorp, College Station, Texas). All statistical tests were two-tailed. We deemed statistical significance at a=0.05.

7.4.1.8.2 Results

For the derivation of the score for stroke recurrence, the total of the 884 consecutive ESUS patients, registered in our dataset, were included. As previously described, 43.3% of them were women, their median age was 67 years, interquartile range [IQR] 54.5–77) and they were followed for a median of 23.7 months corresponding to an overall follow-up period of 2,899 patient-years. Among them, 108 patients (12.2%) had a stroke recurrence,

corresponding to 3.73 (95% CI 3.1–4.5) stroke recurrences per 100 patient-years. The baseline characteristics of patients are summarized in **tables 10 and 22**.

Derivation, prognostic performance, and internal validation of the score

During the bootstrap resampling procedure, the following measures were selected in >80% of the loops and were included in the multivariate model: age, underlying malignancy, leukoaraiosis, multiterritorial infarct, peripheral artery disease, presence of carotid plaques ipsilateral to the index infarct, aortic stenosis, white blood cell count at index stroke, and coronary artery disease. Among them, 3 covariates were identified as independent predictors of stroke recurrence and were included in the score: age, leukoaraiosis, and multiterritorial infarct. The β-coefficients of the covariates included in the final model and the points assigned to each covariate in the final score are presented in **table 23**. The tolerance of the covariates in the final multivariate model ranged between 0.865 and 0.992, and the mean VIF was 1.1 (range 1.01–1.16). The median score value in the derivation cohort was 5 (IQR 3–6, range 0–12). We defined 3 stroke risk groups based on the tertiles of our score distribution; that is, the lower tertile (0–4) indicates lower risk, the medium tertile (5–6) indicates intermediate risk, and the higher tertile (7–12) indicates high risk. The rate of stroke recurrence was 2.10 per 100 patient-years (95% CI 1.44–3.06) in patients with a score of 0–4 (low risk), 3.74 (95% CI 2.77–5.04) in patients with a score of 5–6 (intermediate risk), and 8.23 (95% CI 5.99–11.3) in patients with a score of 7–12 (high risk) **(figure 20)**. Compared to low-risk patients, the risk of stroke recurrence was significantly higher in patients with intermediate risk (HR 1.78, 95% CI 1.1–2.88) and high risk (HR 4.67, 95% CI 2.83–7.7). In Kaplan-Meier analysis, the cumulative probability of stroke recurrence during available follow-up was statistically different among low-risk

(7.6%, 95% CI 4.7–13), intermediate-risk (13.6%, 95% CI 9–20.8), and high-risk (19.4, 95% CI 12.7–33.2) patients (log-rank test $\chi2 = 43.9$, $p < 0.001$) **(figure 21)**. The score was well-calibrated in the derivation cohort, with the Hosmer-Lemeshow test being statistically not significant ($\chi2 = 12.1$, $p = 0.357$) **(figure 22)**. The AUC of the score in the derivation cohort was 0.63 (95% CI 0.58–0.68) **(figure 24).**

External validation

The external validation cohort included 820 patients (43.4% women, median age 67, IQR 53–77) followed for a median of 24 months corresponding to an overall follow-up period of 2,372 patient-years. Among them, 105 patients had a stroke recurrence corresponding to 4.39 (95% CI 3.62–5.31) stroke recurrences per 100 patient-years. The baseline characteristics of patients are summarized in **table 22**. The median value of the score was 6 (range 0–12) and 46.2% of patients were classified as low risk (median score 4), 30.7% as intermediate risk (median score 6), and 23.1% as high risk (median score 8). The score was well-calibrated in the external validation cohort with the Hosmer-Lemeshow test being statistically not significant ($\chi2 = 21.7$, $p = 0.753$) **(figure 22).** An additional calibration plot for predicted estimates of Cox regression analysis and observed events showed satisfactory performance of the score in the external validation cohort **(figure 23)**. Both the intercept ($p = 0.469$) and the slope of the calibration ($p = 0.974$) suggested good correlation of the observed outcomes and the predictions from the score. The AUC of the score in the external validation cohort was 0.60 (95% CI 0.54–0.66) **(figure 24).**

***Table 22**: Baseline characteristics of patients of the derivation and external validation cohorts*

Baseline characteristics	Derivation cohort			External validation cohort		
	Patients who did not have a stroke recurrence n=776	Patients who had a stroke recurrence n=108	p-value	Patients who did not have a stroke recurrence n=715	Patients who had stroke recurrence n=105	p-value
Age (years)	63.7(16.6)	70.3(13.3)	<0.001	63.1(16.9)	68.8(13.8)	<0.001
Female sex (%)	337(43.43)	46(42.59)	0.87	406(56.78)	60(56.07)	0.89
NIHSS at admission	6(10)	6(7)	0.295	4(7)	4(6)	0.803
Hypertension (%)	467(60.18)	79(73.15)	0.009	421(58.88)	62(57.94)	0.854
Dyslipidemia (%)	516(66.49)	69(63.89)	0.592	283(39.58)	59(55.14)	0.002
Smoking (%)	304(39.18)	35(32.41)	0.175	257(36.10)	44(41.51)	0.281
Diabetes (%)	141(18.17)	20(18.52)	0.93	149(20.84)	19(17.76)	0.461
Coronary artery disease (%)	107(13.82)	25(23.15)	0.011	67(9.91)	18(17.82)	0.018
Previous stroke or TIA (%)	122(15.72)	27(25.00)	0.016	71(9.93)	33(30.84)	<0.001
Leukoaraiosis (%)	144(18.56)	29(26.85)	0.042	277(39.29)	51(49.04)	0.059
Multiterritorial infarct (%)	35(4.51)	10(9.26)	0.035	100(14.16)	20(19.05)	0.189
Antiplatelet at discharge (%)	709(92.92)	101(95.28)	0.365	647(95.99)	97(94.17)	0.394
Anticoagulant at discharge (%)	60(7.86)	6(5.66)	0.422	54(7.55)	11(10.28)	0.329

***Table 23:** Multivariable model of predictors of stroke recurrence in patients with ESUS and derivation of the score*

Covariate	HR (95%CI)	Coefficient (95%CI)	p-value	Score points
Age (per increasing decade after 35 years of age)	1.37 (1.16-1.61)	0.311 (0.148-0.474)	<0.001	1 point per every decade after 35 years of age
Leukoaraiosis	1.89 (1.20-2.98)	0.636 (0.181-1.09)	0.006	2
Multi-territorial infarct	2.47 (1.28-4.76)	0.903 (0.245-1.56)	0.007	3

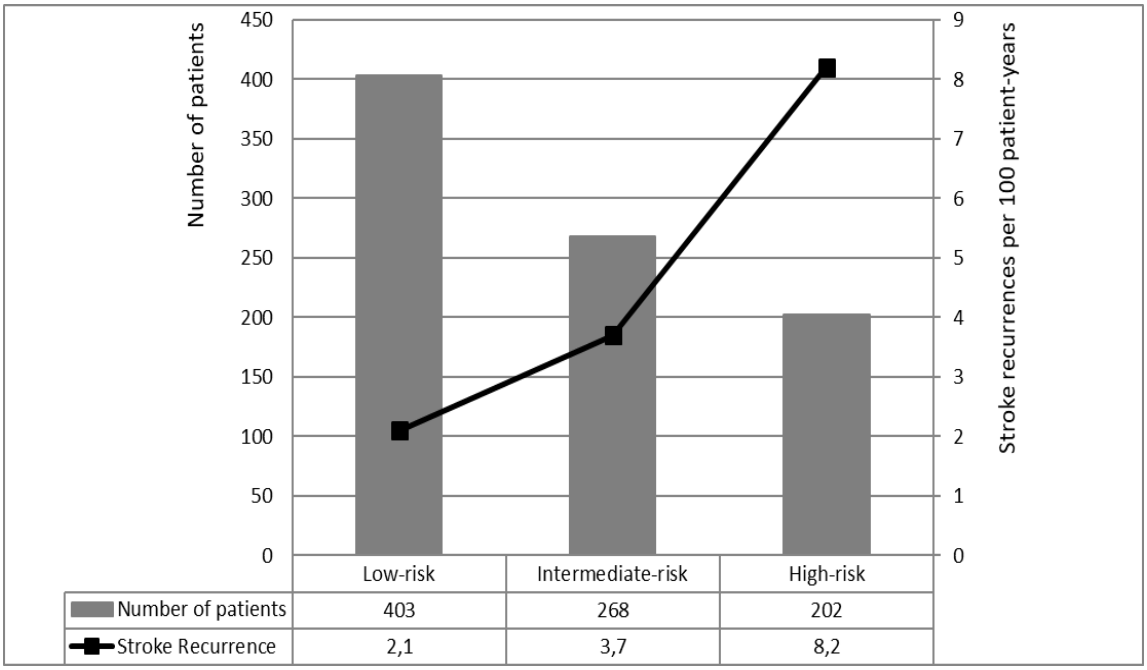


Figure 20 : *Number of patients and annualized rates of stroke recurrence per risk category in the derivation cohort*

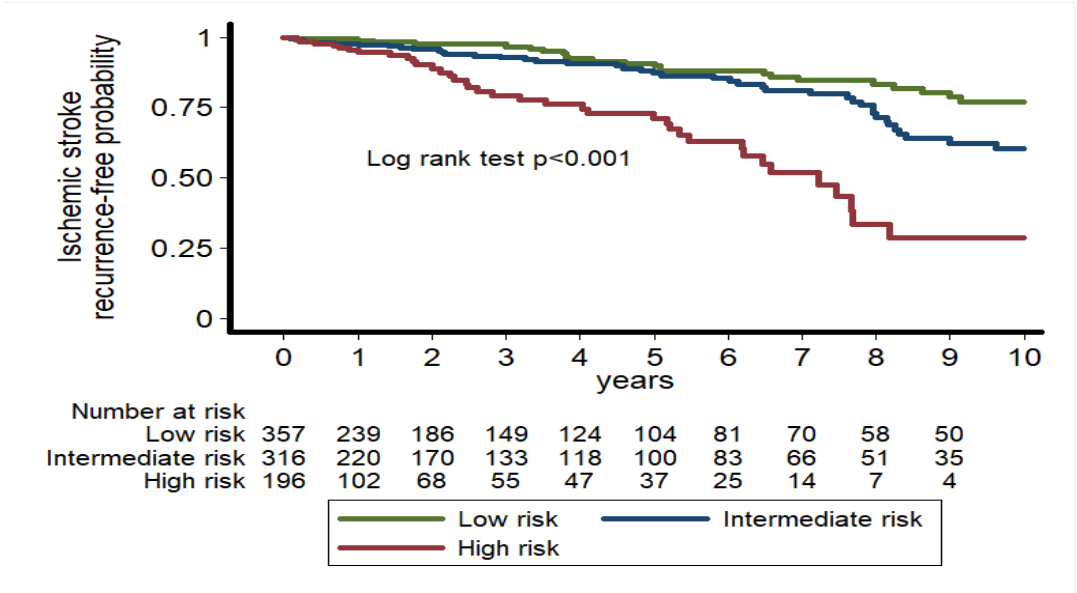


Figure 21 *: Kaplan-Meier curves of cumulative probabilities of stroke recurrence across risk categories in the derivation cohort*

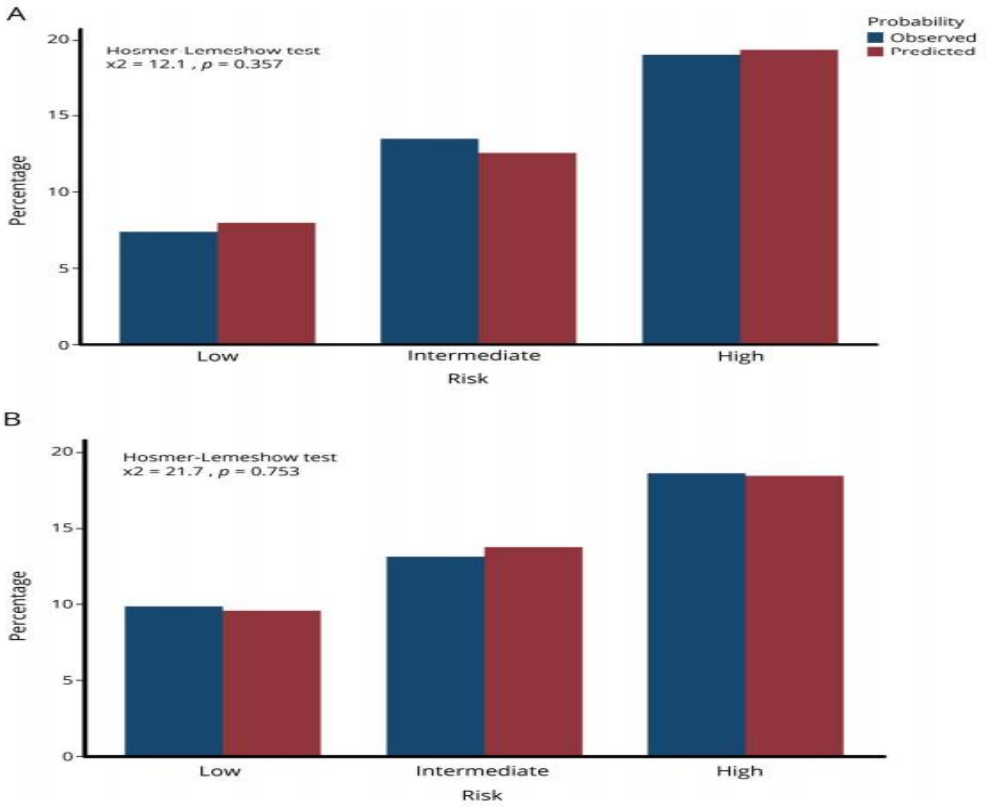


Figure 22 : *Model calibration in the derivation (A) and external validation (B) cohorts*

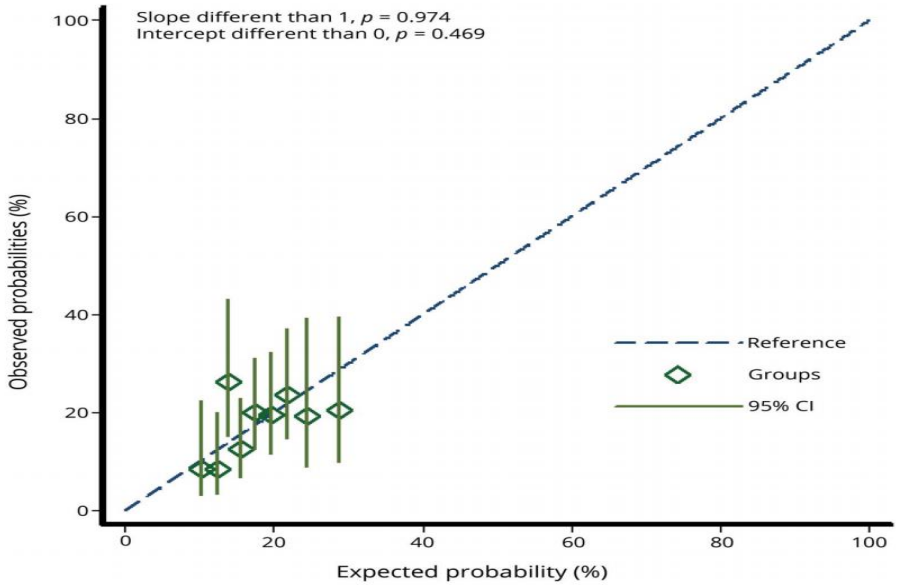


Figure 23*: Calibration plot of observed (y-axis) and predicted survival probabilities (x-axis) in the external validation cohort*

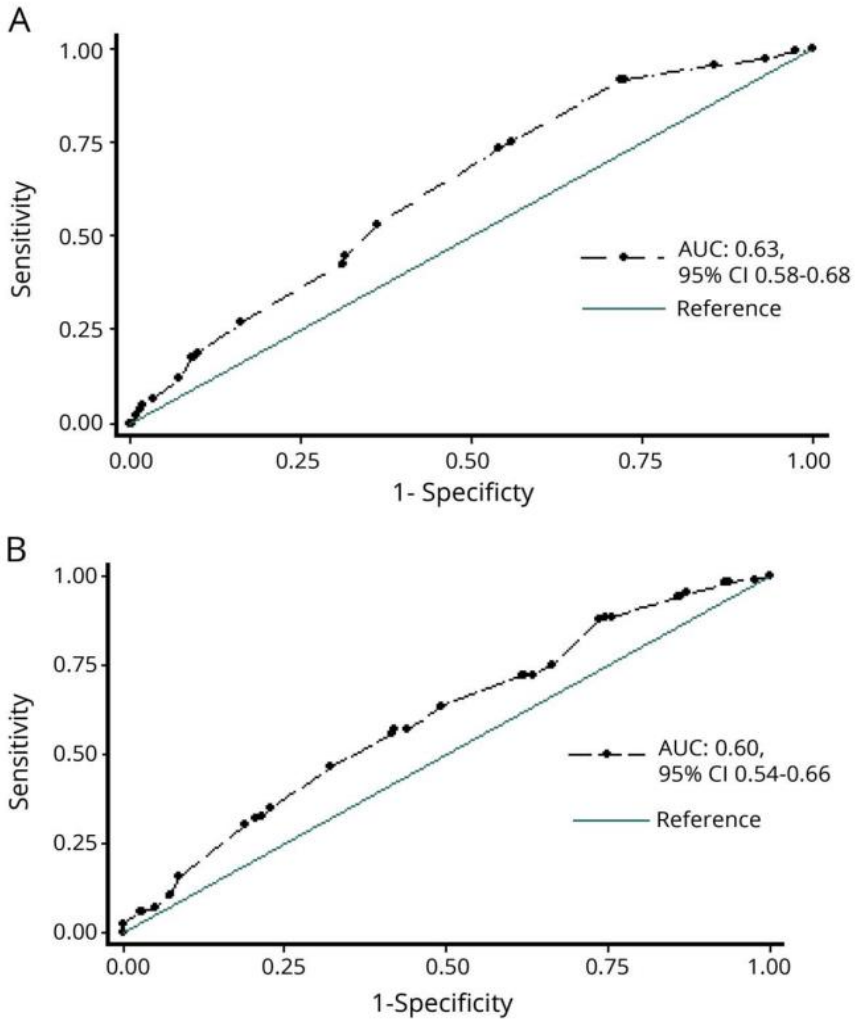


Figure 24 : *ROC curves in the derivation (A) and the external validation (B) cohorts*

7.4.2 Death during follow-up

Among the 884 registered patients, during the median follow-up of 23.7 months, corresponding to an overall follow-up period of 2,899 patient-years, death occurred in 165 patients (18.7%).

7.4.2.1 ESUS severity and risk of death

The annualized rate of death was 5.4% in patients with severe ESUS (admission NIHSS ≥ 6) and 3.7% in patients with mild ESUS (admission NIHSS < 6) (unadjusted HR: 1.46, 95%CI: 1.03–2.06) **(table 16).** In the multivariable regression analysis, the risk for death was higher in patients with severe ESUS compared with mild ESUS (HR: 1.51, 95%CI: 1.05–2.16, p = 0.025). The 10-year cumulative probability of death was higher in patients with severe ESUS compared with mild ESUS (40.5% (95%CI: 32.5–50.0) vs. 34.0% (95%CI: 26.0–43.6) respectively; log-rank test: 4.54, p=0.033; **Figure 25).**

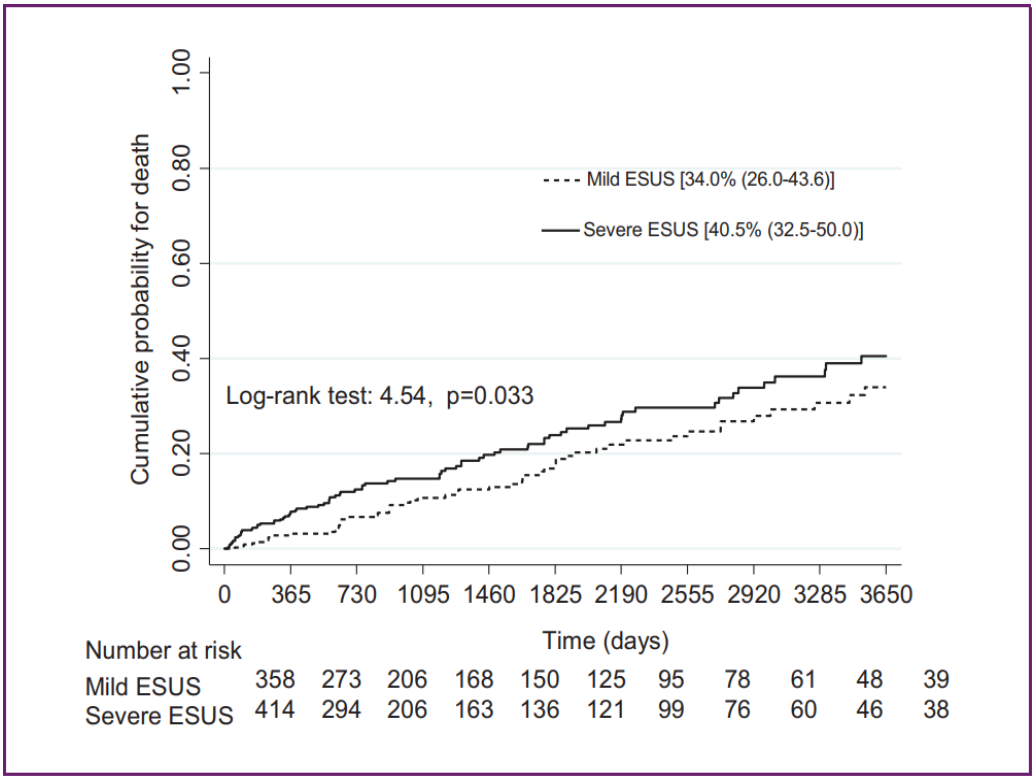


Figure 25 : *Ten-year cumulative probability of death in patients according to ESUS severity*

7.4.2.2 SVE and Risk of Death

Death occurred in 149 (17.5%) of patients in the overall population during follow up. The annualized rate of death was 3.9% in patients with no SVE, 5.8% in patients with greater than 0-1 SVEs, 11.2% in patients with greater than 1-2 SVEs and 9.2% in patients with greater than 2SVEs **(Figure 7)**. In the multivariate regression analysis, the number of SVEs was not associated with the risk of death **(Figure 8 lower panel).** The 10-year cumulative probabilities of death were 30.3% (95%CI: 23.2-37.4), 43.4% (95%CI:27.1-59.7), 70.7% (95%CI: 52.7%-88.7%) and 48.9% (95%CI: 32.8%-65.0%) in patients with no SVE, greater than 0-1, greater than 1-2 and greater than 2 SVEs respectively **(Figure 9 right panel).**

7.4.2.3 Nonstenotic carotid plaques and Risk of death

The annualized rate of death was 5.5% in the overall population with available information about the presence or absence of ipsilateral nonstenotic carotid plaques, occurring in 146 patients. The annualized rate of death was 5.0% in patients with ipsilateral nonstenotic carotid plaques (occurring in a total of 51 patients) and 5.7% in patients without (occurring in a total of 95 patients) (unadjusted HR 0.86, 95% CI 0.61–1.21). In Kaplan-Meier analysis, the 10-year cumulative probability of death was not statistically different between patients with ipsilateral nonstenotic carotid plaques and those without (41.5%, 95% CI 33.9–49.1; 38.4%, 95% CI 26.8–50.0, respectively, log-rank test 0.727, p = 0.394, **figure 26**).

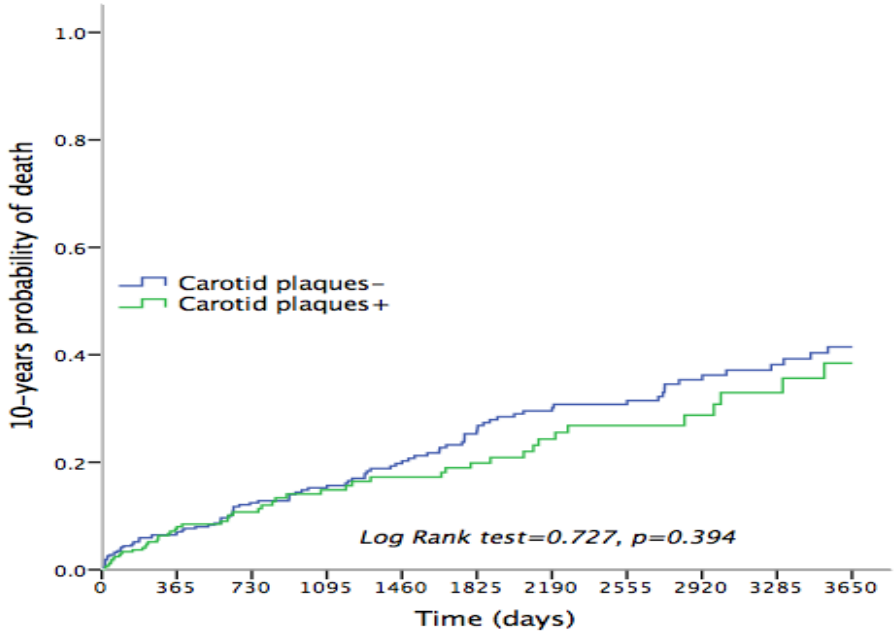


Figure 26: *10-year cumulative probability of death in patients with and without non-stenotic carotid plaques ipsilateral to the index stroke*

7.4.3 Performance of the HAVOC score in our cohort

The HAVOC score (hypertension, age, valvular heart disease, peripheral vascular disease, obesity, congestive heart failure, and coronary artery disease) was proposed as a clinical score for the prediction of AF in patients with cryptogenic stroke or transient ischemic attack. It assigns 2 points to hypertension, 2 points to age ≥75 years, 2 points for valve disease, 1 point for peripheral vascular disease, 1 point for obesity, 4 points for congestive heart failure, and 2 points for coronary artery disease. It was developed and internally validated using data from 1995 to 2015 in Stanford Translational Research Integrated Database Environment and showed good model discrimination (area under the curve, 0.77). In particular, only 2.5% of patients with a low-risk HAVOC score (ie, 0–4)

were diagnosed with new incident AF during follow-up (169). It was also externally tested in the CRYSTAL-AF cohort (Cryptogenic Stroke and Underlying AF trial), where AF was detected in 18.5% of patients with a low-risk HAVOC score (ie, 0–3) (237).

Statistical Analysis

To assess the performance of the score, we examined its discriminatory power and calibration in our dataset (226). Discrimination was defined as the degree to which the prognostic score enables the discrimination between patients with favorable and unfavorable outcome and was assessed by the calculation of the area under the curve. Calibration was defined as the agreement between predicted and actual outcome and was assessed with the use of the Hosmer-Lemeshow goodness of-fit test with 10 groups. We also assessed the specificity, negative predictive value, and accuracy of low-risk HAVOC score (ie, 0–4, as proposed in its original publication (169)) to identify patients without new incident AF during follow-up. The specificity (or else, true negative rate) was defined as the probability that a patient has a score of 0 to 4 if new incident AF is not diagnosed during follow-up and was calculated with the following formula: true negative/(true negative and false positive). The negative predictive value was defined as the probability that new incident AF is not diagnosed during follow-up if the score the patient has a score of 0 to 4 and was calculated with the following formula: true negative/(false negative and true negative). Accuracy was defined as the overall probability that a patient will be correctly classified and was calculated with the following formula: (true negative and true positive)/(true negative and false negative and true positive and false positive). There was no imputation of missing data. Patient characteristics were described by groups using

proportions for discrete variables and medians with interquartile range for continuous. Group differences were summarized by reporting the odds ratio (OR) and 95% CI. Statistical analyses were performed with R package (version 3.5.3).

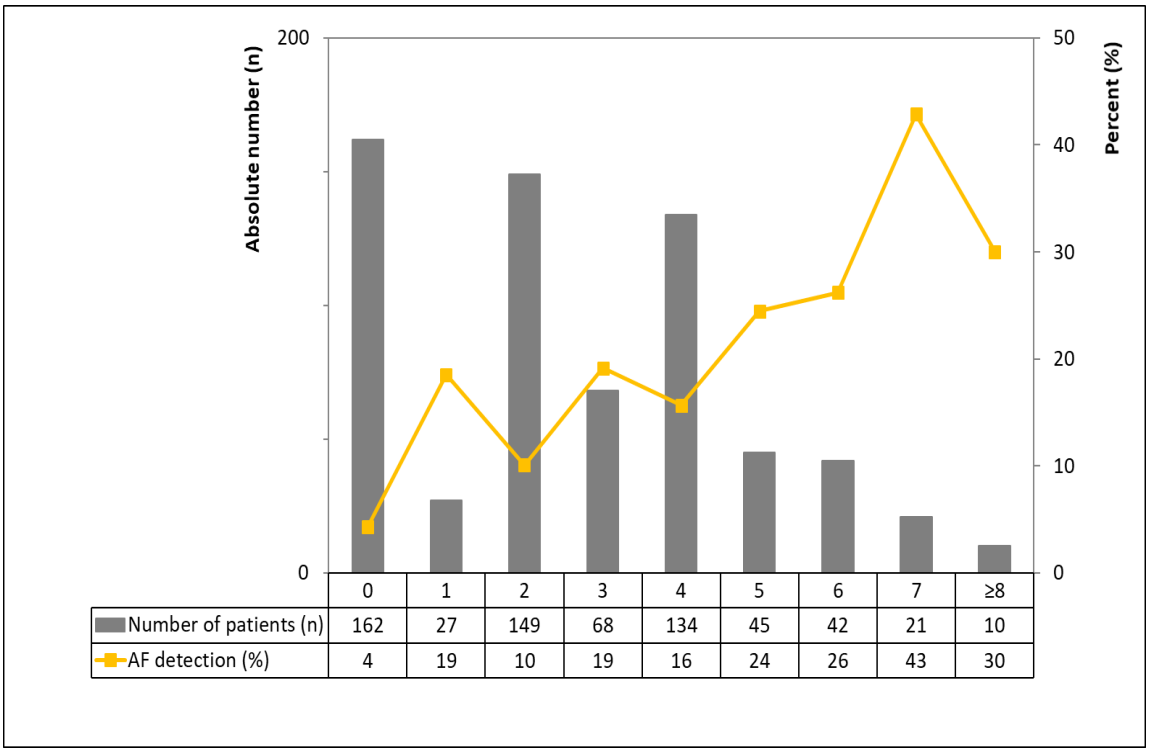
Results

From the 884 patients in our dataset, 658 patients had all the required parameters for the assessment of the HAVOC score and were included in the analysis. In these 658 patients with ESUS (median age: 67 years, 44% women), the median HAVOC score was 2 (interquartile range, 3). There were 540 patients (82%) with a HAVOC score 0 to 4 and 118 (18%) with a score ≥5. Only 2 patients (0.4%) had a HAVOC score ≥10. The baseline characteristics of patients per HAVOC score are summarized in **Table 24**.

Table 24 : *Baseline patient characteristics per HAVOC score.*

Variable	HAVOC (0-4) (n = 540)	HAVOC (≥5) (n = 118)	Odds-ratio	95% Confidence intervals	p-value
Female sex	244 / 540 (45.2%)	49 / 118 (41.5%)	0.86	0.57 - 1.29	0.4
Age (years)	64.7 (23.2)	77.4 (12.5)	1.08	1.06 - 1.10	<0.001
NIHSS	6.0 (9.0)	6.0 (7.5)	1.00	0.96 - 1.02	0.7
Hypertension	304 / 540 (56.3%)	113 / 118 (95.8%)	17.54	7.80 - 50.22	<0.001
Dyslipidemia	356 / 540 (65.9%)	90 / 118 (76.3%)	1.66	1.06 - 2.67	0.03
Diabetes	77 / 540 (14.3%)	36 / 118 (30.5%)	2.64	1.66 - 4.16	<0.001
Smoking	219 / 540 (40.6%)	39 / 118 (33.1%)	0.72	0.47 - 1.10	0.13
Coronary artery disease	28 / 540 (5.2%)	50 / 118 (42.4%)	13.45	8.00 - 23.04	<0.001
Previous stroke	97 / 540 (18.0%)	21 / 118 (17.8%)	0.99	0.58 - 1.64	0.9
New incident atrial fibrillation during follow-up	61 / 540 (11.3%)	34 / 118 (28.8%)	3.18	1.96 - 5.11	<0.001

The overall-follow-up was 2340 patient-years corresponding to 3.6 years per patient. During follow-up, new incidental AF was diagnosed in 95 patients (14.4%). The number of patients and the rate of new incident AF during follow-up per HAVOC score are presented in **Figure 27**. The rate of new incident AF was 28.8% among the 118 patients with a HAVOC score of ≥5 and 11.3% among the 540 patients with a HAVOC score of 0 to 4 (age- and sex-adjusted OR, 2.29 [95% CI, 1.37–3.82]). The rate of new incident AF in patients with a HAVOC score of 0 was 4.3%.



	0	1	2	3	4	5	6	7	≥8
Number of patients (n)	162	27	149	68	134	45	42	21	10
AF detection (%)	4	19	10	19	16	24	26	43	30

***Figure 27** : Number of patients and rate of new incident AF during follow-up per HAVOC score*

The specificity (or else, true negative rate) of low-risk HAVOC score (ie, 0–4) to identify patients without new incident AF during follow-up was 88.7%; that is, 11.3% of patients with low-risk HAVOC score were diagnosed with new incident AF during follow-

up. The negative predictive value of low-risk HAVOC score, or else the probability that new incident AF will not be diagnosed if the HAVOC score is 0 to 4, is 85.1%. The accuracy was 78.0%. The area under the curve of the HAVOC score to predict new incident AF during follow-up was 68.7% (95% CI, 62.1%–73.3%). The Hosmer-Lemeshow X^2 value of the logistic model relating new incident AF at follow-up with HAVOC score adjusted for age and sex was estimated at 14.44 (P=0.07), showing that the fit of the model to the observed data was of borderline acceptance.

8 Discussion

The main intention of this research was to develop a clinical tool that can assist the identification of ESUS patients with low or high probability of new incident AF, in order to support a strategy of more personalized allocation of the available resources for prolonged cardiac monitoring. The result was the generation of a score, the AF-ESUS score, consisting of eight, readily available parameters (age, arterial hypertension, left atrium dilatation, left ventricular hypertrophy, low left ventricular ejection fraction, supraventricular extrasystoles, subcortical infracts and presence of non-stenotic carotid plaques). Among the 42.3% of the population who had a score of ≤0, only 1.97% had new incident AF during follow-up of two years. The relative risk of new incident AF was 13 times higher in patients with a score of >0 compared to patients with ≤0. The sensitivity and the negative predictive value of a score of ≤0 for new incident AF during follow-up were 94.9% (89.3–98.1%) and 98.1% (95%CI: 95.9–99.1%), respectively. The score was internally validated and its performance was further assessed in a small independent cohort of consecutive ESUS patients with prolonged cardiac monitoring.

As it has already been mentioned, AF is frequently detected in patients with ESUS during follow-up, and it seems that the rate of AF detection correlates with the duration of cardiac rhythm monitoring (8–10). Although it is datable whether the detected AF episodes are the real underlying cause of ESUS, especially if they are short-lasting or detected long after the index stroke (238), still these episodes are the only main indication for oral anticoagulation in these patients, given the negative results of the 2 recent randomized control trials, NAVIGATE and RESPECT- ESUS. Thus, prolonged cardiac monitoring

beyond 24h is still recommended by the latest guidelines, while a recent white paper of the AF-SCREEN International Collaboration, recommended that patients with ischemic stroke or TIA who do not have a prior AF diagnosis, should have continuous electrocardiogram (ECG) monitoring after a stroke for at least 72 h (239). However, as data from international stroke registries show, in routine clinical practice, this can be achieved only in the minority of ESUS patients, mainly due to limited resources (12). The present research suggests a score as a clinical tool which could potentially guide a strategy of selecting patients for more extended cardiac monitoring: in our cohort, a score of ≤0 identified a large proportion of ESUS patients (>40% of the overall cohort) who had low probability of new incident AF, a finding which suggests that patients with higher scores may be better candidates for prolonged automated cardiac monitoring. It is likely that this strategy could be associated with more efficient use of the available resources and hence, could be more costeffective and increase their diagnostic yield. However, it should be stressed that the proposed score should not be used to guide treatment decisions with regard to anticoagulation in these patients. According to current evidence, patients with ESUS should receive antiplatelet treatment.

The proposed score comprises of a set of eight covariates (age >60 years, arterial hypertension, left atrial dilatation, any supraventricular extrasystole, left ventricular ejection fraction <35%, left ventricular hypertrophy, subcortical infracts and presence of non-stenotic carotid plaques) which means that convey clinical, echocardiographic, electrocardiographic, brain and vascular imaging information about the patient. Age >60 years, arterial hypertension, left atrial dilatation and presence of supraventricular extrasystoles came out to be associated with a higher risk of new incident AF whereas low

left ventricular ejection fraction, left ventricular hypertrophy, subcortical infract and the presence of non-stenotic carotid plaques were associated with lower risk of AF detection.

As in our study, *increasing age* has been strongly and consistently correlated with higher risk of AF in the general population, as well as in both the unselected and the ESUS stroke population.

Arterial hypertension has been also associated strongly with the risk of AF through several different mechanisms like the promotion of left ventricular fibrosis which can sequentially lead to left ventricular hypertrophy, left ventricular stiffness and impairment of the left ventricular diastolic function (240). These alterations may increase left atrial stretch and pressure and hence provoke structural, contractile, and electrophysiological changes of the left atrium, ultimately predisposing to AF (240). However, it should be noted that left atrial remodelling is only one of the possible pathways though which arterial hypertension predisposes to AF as the total of the different pathogenetic mechanisms explaining the higher propensity of hypertensive patients to develop AF is still unknown. This is compatible with the fact that, in the proposed score, left atrial dilatation remained a significant predictor of new incident AF independent of arterial hypertension.

Left atrial dilatation is also a well-established predictor of AF in the general population as well as in patients with ischemic stroke, including patients with ESUS and there is a linear association between left atrial enlargement and the probability to detect AF (182). However, the optimal threshold of left atrial diameter (LAD) that could be used clinically to select patients for long term cardiac monitoring remains to be established: if the LAD threshold is placed high, the specificity would be high but the sensitivity would be low depriving many patients from getting long-term cardiac monitoring despite

significant probability of AF. On the other hand, if the LAD threshold is placed low, many patients would get long-term cardiac monitoring despite having a relatively low probability of AF detection, thus undermining the costeffectiveness of this diagnostic approach. In the stepwise regression for the development of the multivariate model for AF detection, we used the threshold of 40mm for left atrial diameter but it was not a random choice as, in a separate analysis of our ESUS dataset, we compared the prognostic performance of different LAD thresholds to predict new incident AF during follow-up and we found that the LAD threshold with the highest prognostic performance was 40mm, which is also the threshold widely used in clinical practice to describe left atrial enlargement (241). The negative prognostic value of this threshold to predict new incident AF was 0.92, i.e. in ESUS patients with LAD $\leq$ 40 mm, the probability that new incident AF will not be diagnosed during a follow-up of 3.6 years is 92%.

In the context of the previous analysis, aiming to provide evidence about the prognostic performance of different LAD thresholds to predict AF, we also assessed the association between LAD>40mm and stroke recurrence during follow up in our dataset. Despite the association between LAD and AF detection and the strong association between AF and ischemic stroke, our study did not detect an association between LAD and stroke recurrence. A plausible explanation could be that the detection of AF is an indication for initiation of oral anticoagulation instead of antiplatelet treatment, a treatment decision which reduces stroke risk significantly. This may not apply to some patients for whom AF is diagnosed during admission for a stroke recurrence, however, only a third of AF episodes after an ESUS are diagnosed during a stroke recurrence (5).

It should be emphasized that, although left atrial volume is a more accurate marker of atrial size and a better predictor of incident AF compared with left atrial diameter, in our analysis we used left atrial diameter for the definition of left atrial enlargement as left atrial volume was not reported for a large number of patients.

Any supraventricular extrasystole recorded during all standard 12-lead ECGs performed during hospitalization for the ESUS was the fourth covariate that came out to be associated with an increased risk for AF detection in our multivariate model. Supraventricular extrasystoles (SVE) are common in patients with acute ischemic stroke (242) and have a well-established association with the probability of AF detection in the general population and in patients with cryptogenic stroke (215,243–248). In the vast majority of these studies, SVEs were assessed during 24-hour Holter monitoring or prolonged cardiac monitoring (243,245–248). On the contrary, very few studies assessed the association between SVEs recorded during short-term ECG recordings like the standard 12-lead electrocardiogram (ECG) and new AF, and none in ESUS patients, as in the present study (215,244). In fact, in the separate analysis that we conducted in order to assess the association between SVEs and the probability of AF detection during follow-up, we classified patients in 4 groups according to the number of SVE per 10 seconds of all available ECG during hospitalization (no SVE, greater than 0-1 SVEs, greater than 1-2 SVEs, and greater than 2 SVEs) and we concluded that the number of SVEs was indepedently associated with the probability of AF detection. The negative prognostic value of the presence of any SVE for the prediction of AF detection was more than 91%, ie in patients with no SVEs, the probability that AF will not be detected during a follow-up of 3.4 years is more than 91%. These findings support the hypothesis that the presence of SVEs on standard 12-lead ECG is a significant predictive factor for AF detection that could

complement decision making about the selection of patients for prolonged rhythm monitoring in ESUS.

It should be mentioned that, during the data collection, we recorded all available parameters from in- or/and outpatient long-term cardiac monitoring, including the total number of SVEs. However, we did not include this variable in the analysis firstly because there were a lot of missing data, and secondly and most importantly because, based on the retrospective design of the study, we could only review the reports of Holter or other prolonged cardiac monitoring examinations and not review and interpret the examinations proper, and it is true that the different number of SVEs given in the reports are usually based on the data given by the machine, which are often unreliable because of artefacts and bad quality recordings. On the contrary, the number of SVEs in the standard 12-lead ECGs performed during hospitalization were accurately measured by reviewing and interpreting all available ECGs found in patient's medical archives. We believe that, including data from short ECG recordings rather than from prolonged cardiac monitor devices, is not a limitation but a strength of our study as it provides evidence that the presence of SVE on standard 12-lead ECGs during hospitalization or routine outpatient clinic visits in patients with ESUS could serve as a simple, low-cost and universally available additional screening tool for further cardiac monitoring.

In the aforementioned analysis concerning SVEs, we also assessed their association with ischemic stroke recurrence and death. Several studies have identified an association between SVEs and risk of stroke and cardiovascular death (248). An analysis of the Copenhagen Holter Study concluded that excessive supraventricular ectopic activity, defined as the presence of either greater than or equal to 30 premature atrial contractions

(PACs)/hour daily or any runs of greater than or equal to 20 PACs detected on 48-hour ambulatory ECG, was associated with an increased risk of ischemic stroke beyond manifest AF (243). An analysis of the Reasons for Geographic and Racial Differences in Stroke Study (REGARDS) concluded that PACs detected on the routine ECG are associated with an increased risk for nonlacunar ischemic strokes, especially in women (215). Also, an analysis of ischemic stroke patients treated at a Stroke Unit in Denmark showed that excessive premature atrial complexes were associated with a higher risk of recurrent stroke or death (249). Finally, the Ibaraki Prefectural Health Study (IPHS) and the Cardiovascular Health Study (CHS) concluded that the presence of supraventricular premature complexes was associated with increased risk of cardiovascular death in the general population (244,245). Our findings in ESUS patients did not identify an association between SVEs and the risk for recurrent ischemic stroke or death. A likely explanation is that our study was underpowered to detect this association, especially taken into consideration the strong trends that were identified for both outcomes in patients with greater than 1 SVEs. Another possible explanation relates to the adjustment of our results with a large number of covariates including demographic, clinical, echocardiographic, carotid ultrasound and acute brain imaging parameters; these covariates were not adjusted for in the aforementioned studies. In addition, the detection of AF might have been a reason for anticoagulation and thus, stroke/death incidence might have been reduced, and the natural history and association of SVE with stroke/death might have been disturbed. However, one cannot exclude the possibility that there is indeed no association.

Low left ventricular ejection fraction and *left ventricular hypertrophy* are 2 parameters that, in the proposed score, were associated with lower risk of new incident AF. This is an interesting finding which may seem irrational given the well-known association

between heart failure and AF risk. However, this finding could be explained by the established etiologic association between heart failure and ischemic stroke: in other words, low left ventricular ejection fraction ejection fraction (which practically corresponds to heart failure with reduced ejection fraction) and left ventricular hypertrophy (which practically corresponds to heart failure with preserved ejection fraction) as pathologies, are considered as potential embolic sources in ESUS, as they could provoke thrombus formation and subsequently embolism in patients, regardless of AF presence. This means that covert AF and these pathologies are competing etiologies of ESUS, something that is compatible with our results (1,35).

Subcortical infarcts were inversely associated with new incident AF diagnosis in our study, a finding which is compatible with previous evidence indicating that AF is correlated with cortical involvement (166,167).

Finally, the presence of *non-stenotic carotid plaques* was inversely associated with the probability of new incident AF in our study. Similar to the case of left ventricular hypertrophy and low left ventricular ejection fraction, this is explained by the fact that non-stenotic carotid plaques and AF are competing etiologies of the ESUS (1,35). This hypothesis is supported by several studies that highlighted the significance of non-stenotic carotid plaques as a cause of ischemic stroke (250,251) especially in the case of irregular or complicated plaques (68,216,252). Complicated American Heart Association (AHA) lesion type VI plaques in the carotid arteries of patients with cryptogenic stroke were found in 37.5% of cases ipsilateral to the stroke, whereas there were no AHA type VI plaques contralateral to the stroke (68). Similar results were also reported elsewhere (252). Furthermore, approximately 20% of patients with cryptogenic stroke had carotid plaque

with intraplaque high-intensity signal ipsilateral to the ischemic stroke compared to the contralateral side (216). These observations underline the potential high risk of nonstenotic plaques based on morphologic and biological characteristics beyond the stenosis degree, suggesting a role in the stroke mechanisms.

It should be noted that, during the data collection, we tried to record information about plaques' consistency (calcified/non calcified or mixed plaques) but, unfortunately, we could not include these parameters in our analysis due to a lot of missing data. This is mainly due to the fact that, based on the retrospective nature of the study, relevant data was assessed by reviewing the reports of ultrasound examinations or CT angiography or magnetic resonance angiography or digital subtraction angiography and not by reviewing the raw images.

In addition, it should be stressed that in the analysis for the derivation of the multivariate model and subsequently the score, we used data about the presence of non-stenotic carotid plaques either ipsilateral or contralateral to ischemic territory. However, in the separate analysis we conducted in order to investigate the association between the presence of only the ipsilateral nonstenotic carotid plaques and the rate of detection of atrial fibrillation, we also found a significant association with lower probability for AF detection (adjusted HR 0.57, 95% CI 0.34–0.96, p = 0.03).

Overall, the present study, which included a large number of well-defined ESUS patients, emerged a score, the AF-ESUS score, consisting of 8 parameters (age >60 years, arterial hypertension, left atrial dilatation, any supraventricular extrasystole, left ventricular ejection fraction <35%, left ventricular hypertrophy, subcortical infracts and presence of non-stenotic carotid plaques), as a prognostic tool for the prediction of AF in ESUS

patients. Several attempts were previously made to develop prognostic tools to predict AF in stroke patients (166,167,169,189,197,198,200,253,254). However, in the majority of these studies, these tools were developed in the unselected ischemic stroke population (166,189,197,198,200,253). Only 2 small studies included patients with cryptogenic stroke (167,254), while only one recent study included about 300 ESUS patients proposing a score consisting of only 2 parameters (age and left atrial enlargement) (203). Another score that has been recently proposed for the prediction of AF in patients with cryptogenic stroke or transient ischemic attack, including several, mainly clinical, parameters, is the HAVOC score (169). As already mentioned, the HAVOC score, consisting of 7 covariates (hypertension, age, valvular heart disease, peripheral vascular disease, obesity, congestive heart failure, and coronary artery disease), was developed and internally validated using data from 1995 to 2015 in the Stanford Translational Research Integrated Database Environment (STRIDE) showing good model discrimination (area under the curve, 0.77) (169).

In the context of our study, we attempted to assess the performance of the HAVOC score using our AF- ESUS dataset as an external independent cohort of patients. The result was that the HAVOC score performed less well than it did in the cohort of its original publication: the area under the curve in our cohort was 68.7% (compared with 77% in the original publication), the negative prognostic value was 85.1% (compared with 97% in the original publication), and the accuracy was 77% (compared with 80% in the original publication) (169). Of importance, 11.3% of patients with a low-risk HAVOC score (ie, 0–4) were diagnosed with new incident AF during follow-up in our cohort, compared with 2.5% in its original publication (169). Our results are in line with a recent analysis of the HAVOC score at the CRYSTAL-AF cohort, in which 18.5% of patients with low-risk

HAVOC score (ie, 0–3) were detected with AF and the negative prognostic value and accuracy were 84.7% and 73.8% respectively (237). These 2 analyses of the HAVOC score at the AF-ESUS (present analysis) and the CRYSTAL-AF cohorts do not confirm the previously reported low rate of AF among ESUS patients with low-risk HAVOC score. As already pointed out, and will be discussed in more detail below, embolism in ESUS patients may be etiologically associated with several conditions like aortic or carotid atherosclerotic plaques causing low-degree stenosis (ie, <50%) covert AF, atrial cardiopathy including other non-AF supraventricular arrhythmias and structural abnormalities of the left atrium, pathologies of the left ventricle, cardiac valvular pathologies, paradoxical embolism through patent foramen ovale or other shunts, cancer and others (1,4). The HAVOC score includes information only for some of these potential causes, which could offer a plausible explanation for the findings of the present and the CRYSTAL-AF cohort analysis. It may be possible that a prognostic tool incorporating information about these parameters could identify more reliably those ESUS patients who are at low risk for AF.

A strength of our main study is that the AF-ESUS score comprises of several clinical, echocardiographic, electrocardiographic, brain imaging, and vascular imaging parameters, which convey a holistic set of information that can support a personalized strategy in the quest of the most appropriate ESUS patients for long term cardiac monitoring. The AF-ESUS score is further strengthened by the related literature which provides solid pathophysiologic evidence for the association of the score's covariates with AF, as described in detail above. Another strength of the study is that all covariates which are included in the AF-ESUS score, are readily available within the standard diagnostic workup required to classify a patient as ESUS (i.e. echocardiography, standard 12-lead ECG, brain imaging and carotid imaging), and therefore, no further work-up is necessary

for the calculation of the score. However, it should be noted that echocardiography may still be difficult to get in a timely manner in health systems with limited resources. Also, another strength of the AF-ESUS score is the proposed threshold of ≤0, which has high sensitivity and high negative predictive value to identify a large proportion of patients (>40% of the overall population) who have low probability of new incident AF. Other strengths of the study include the large number of consecutive, well-defined ESUS patients, and its multicenter design.

Our patients were not systematically screened with prolonged cardiac monitoring. Hence, it is possible that some AF episodes may have been missed resulting in underestimation of the actual rate of AF. Indeed, the proportion of patients with new incident AF in our ESUS cohort (14.9% during a median follow-up of 24.3 months) was lower compared to a recent real-world cohort of cryptogenic stroke patients with insertable cardiac monitoring (21.5% at 24 months, when the AF episode duration threshold was set at ≥2 min) (255). It is possible that missed AF episodes in our study were mostly the short-lasting or subclinical AF episodes; this hypothesis seems plausible taking into consideration that the proportion of AF detection in the aforementioned study became comparable to our study when the AF episode duration threshold was set at ≥60 min (16.2% at 24 months) (255). There is growing evidence that episodes of subclinical AF carry a lower risk for stroke, especially if their duration is only seconds to a few hours (113,114,256). Moreover, it is not known whether anticoagulation decreases the risk of stroke in patients with short episodes of subclinical AF, a question which is currently addressed in randomized controlled trials (257,258). In this context, it seems likely that our study mostly identified longer, clinically more relevant episodes of AF which carry a well-known risk of ischemic stroke and have a strong therapeutic indication for oral anticoagulation, rather than short

episodes of subclinical AF with currently unclear clinical and therapeutic implications (7,34). This belief is largely confirmed by the result of the external validation of the score in a well-defined ESUS population monitored with ILR. In fact, when assessing the performance and reliability of the AF-ESUS score in an independent cohort of ESUS patients with prolonged cardiac monitoring, we found that ESUS patients with AF-ESUS score ≤ 0 were unlikely to develop long lasting episodes of AF on follow-up and particularly, no patient developed AF episodes lasting more than 10h.

Another possible limitation is that, given the retrospective nature of the study, intracranial vascular imaging was not a prerequisite for inclusion. This approach was also followed in the NAVIGATE-ESUS trial (2) and can be justified in Western populations where intracranial arterial stenoses are not frequent (212). Other limitations include the risk of registration bias within and between the participating registries, the retrospective design of the analysis, and differences in the work-up of patients during the in-hospital phase.

Several markers of atrial cardiopathy have been associated with AF including biomarkers (e.g. N-terminal pro-brain natriuretic peptide (24,55,259), high sensitive cardiac troponin T (201), cardiac MRI (for the assessment of atrial fibrosis) (56), ECG indices (e.g. $PTFV_1$) (57–59), and transesophageal echocardiogram (for the assessment of spontaneous echocardiographic contrast (260) and the morphology of the left atrial appendage) (261) . These markers were not available in our datasets. It may be argued that the inclusion of such markers in our score could have increased further its predictive performance. However, currently, this could be considered impractical and could reduce the applicability of the score, as none of these markers is yet part of the routine clinical practice.

In conclusion, the present study proposes a tool, the AF-ESUS score, that has high sensitivity and high negative predictive value to identify a large proportion of ESUS patients (>40% of the overall cohort) who have low probability of new incident AF according to the prediction of the score. The relative risk of new incident AF was 13 times higher in patients with a score of >0 compared to ≤ 0. The score has acceptable discrimination and calibration performance and has been validated both internally and externally in an independent cohort of ESUS patients with prolonged cardiac monitoring. Inferentially, the AF-ESUS score could potentially assist clinical decisions about the selection of patients for prolonged automated cardiac monitoring and could also potentially guide the design of future trials aiming the enhancement of the optimal therapeutic treatment in selected ESUS patients with higher or lower probability of AF detection.

Another interesting finding deriving from the analysis of our large multicenter dataset of about 800 ESUS patients is that there is major overlap of potential embolic sources in patients with ESUS: in our analysis two thirds of patients with ESUS had at least 2 PES, whereas one third of patients had at least 3 PES. The most prevalent PES were LV disease, arterial disease, and atrial cardiopathy (AC), each one of which was present in nearly half of the study population.

It is likely that our estimate for the degree of overlap of PES is only an underestimate of the actual degree of overlap. As already discussed, given the pragmatic nature of this study, the identification of PES relied on investigations that are routinely performed in clinical practice, like standard 12-lead ECG, transthoracic echocardiogram, extracranial vascular imaging, and automated cardiac rhythm monitoring. It is likely that a larger number of PES might have been identified if all our patients had an exhaustive panel

of investigations that are currently not routinely used in most patients with ESUS. For example, biomarkers (eg, NT-proBNP (262) and high-sensitivity cardiac troponin T (263)), cardiac magnetic resonance imaging (for the assessment of atrial fibrosis (264)), ECG indexes (eg, $PTFV_1$ (60,265)), and transesophageal echocardiogram (for the assessment of spontaneous echocardiographic contrast (266,267) and the morphological features of the left atrial appendage (183)) could have led to more diagnoses of AC; intracranial vascular imaging and transesophageal echocardiogram could have led to more diagnoses of intracranial and aortic atherosclerosis, respectively (73,268); and transesophageal echocardiogram could have identified a larger number of patients with PFO, if performed in all patients regardless of their age. If patients were investigated with such an exhaustive diagnostic workup, it is highly likely that the degree of overlap of PES would be much higher and only a small minority of patients would be considered to have a single or no PES. However, such a diagnostic panel would be considered unrealistic to apply to the entire population with ESUS, even in high-resource settings.

The ESUS concept has been criticized that it promotes the lumping approach (ie, a standardized, one-size-fits-all diagnostic approach aiming to detect the major-risk embolic sources for which there is strong evidence to guide secondary prevention) (269). The results of the present study offer support to this strategy by showing that in a remarkable majority of patients, the strategy of an exhaustive diagnostic workup may be not only unrealistic in terms of availability of resources but also futile, as it would rarely lead to a single PES (for most of which there is low quality of evidence to guide management), but rather to multiple PES and subsequent frustration in the attempt to conclude on the causal one (35).

The NAVIGATE ESUS and the RE-SPECT ESUS (Dabigatran Etexilate for Secondary Stroke Prevention in Patients With ESUS) trials showed that anticoagulation is not superior to aspirin for prevention of stroke recurrence in patients with ESUS, indicating that the lumping therapeutic approach of oral anticoagulation for the unselected population with ESUS was not the optimal strategy and indirectly validating the ESUS concept as an etiologically heterogeneous entity. The heterogeneity of embolic sources and their remarkable overlap, as estimated in the present study, could possibly explain these negative results: for some of the embolic sources (like AC, AF, LV disease, PFO, and cancer), the main pathophysiologic mechanism for thrombogenesis is low blood flow, which predisposes to formation of red thrombi that may respond better to anticoagulation. On the other hand, for other embolic sources, like aortic and nonstenotic carotid atherosclerosis, the ulceration of a plaque triggers the formation of white thrombi that may respond better to aspirin. In this context, it may be hypothesized that treating patients with ESUS with anticoagulation rather than aspirin would just result in exchanging red thrombi for white, with the overall burden remaining largely unchanged. If this hypothesis is correct, it would be rational to expect that simultaneous inhibition of red and white thrombi with a combination of anticoagulation and aspirin would be associated with a significant reduction of stroke recurrences in patients with ESUS. The COMPASS (Cardiovascular Outcomes for People Using Anticoagulation Strategies) trial showed that a combination of low-dose rivaroxaban and aspirin was associated with a large reduction of stroke risk compared with aspirin as monotherapy in patients with stable atherosclerotic vascular disease (270). These thoughts provide a rationale for a randomized controlled trial of combination of anticoagulation and aspirin for the prevention of stroke recurrence in the unselected population with ESUS. Also, this rationale does not apply to patients who are detected with

AF who should be anticoagulated. Furthermore, it may not apply to patients with AC if, in the meanwhile, the ARCADIA (Atrial Cardiopathy and Antithrombotic Drugs in Prevention After Cryptogenic Stroke) trial, which is currently investigating whether oral anticoagulation with apixaban is a better strategy compared with aspirin in patients with AC, reports positive results (60).

Among all potential PES, the AF group was associated with the highest risk of stroke recurrence. Although it is debatable how strong is the causative association between ESUS and episodes of AF detected during follow up, especially if they occur late or are of short duration (238), still it is important that these episodes are detected on time as they may warrant oral anticoagulation, which could reduce the risk of stroke recurrence. Given the large prevalence of ESUS and the restricted resources for prolonged cardiac monitoring, this finding highlight that it is important to develop prognostic tools that may aid to the stratification of the likelihood of AF detection in patients with ESUS.

A limitation of the present analysis is that the estimated degree of PES overlap may be an underestimate of the actual overlap, as it might have been higher if a more extensive panel of diagnostic investigations had been performed, as argued above. However, at the same time, this strengthens further the conclusion of this study that there is remarkable overlap of PES in patients with ESUS. The conclusion of the present analysis may possibly explain the negative results of the NAVIGATE ESUS and RE-SPECT ESUS trials and offer a rationale for a randomized controlled trial of combination of anticoagulation and aspirin for the prevention of stroke recurrence in patients with ESUS.

It is also worthwhile to discuss that, given the extremely advantageous nature of clustering algorithms for exploring the potential underlying etiology in particularly

heterogeneous diseases, like ESUS, we also attempted to investigate the potential sources of embolism in ESUS patients using a data-driven, machine-learning analytical method. The result was the identification of 4 clusters of patients based on their baseline characteristics: the largest cluster which included more than half of the overall population, was associated with the presence of arterial disease; two clusters of medium size including approximately 15-20% of the overall population, were associated with atrial cardiopathy and PFO respectively; and a small cluster which included only 5% of the overall population and was associated with left ventricular disease. Atrial fibrillation was not associated with any cluster. The risk of stroke recurrence was similar across clusters.

The results of this analysis are in accordance with evidence provided by previous studies concerning the importance of the etiological association between ESUS and atherosclerotic plaques (68,250,252,271), atrial cardiopathy (50) and PFO presence (272). This analysis also adds to evidence supporting the argument that AF is not so strongly associated with ESUS (4,5,119) as it was initially believed based on several observational studies and randomized trials showing that AF can be detected in 30% of ESUS patients (8–10).

The main strength of this analysis is its design: the data-driven hierarchical-clustering analysis allowed the categorization of patients into distinct clusters based on their all their baseline characteristics, without pre-specification of variables, and then coupling of these clusters with PES. This is a particularly advantageous method in cases of datasets with large degree of heterogeneity between individuals. The categorization of patients into clusters rather than PES is advantageous and more informative, as there is large overlap of PES in patients with ESUS. For example, the previously discussed analysis in the same

cohort showed that left ventricular disease was present in 54.4% of the overall cohort; however, the present analysis showed that the cluster which was associated with left ventricular disease included only 5% of the overall cohort. This suggests that for the majority of patients with left ventricular disease, this would represent an innocent by-stander rather than the actual embolic source. However, the clustering algorithms are empirical methods, which may be limited by the sample size of the data and number of clinical features collection to determine cluster associations, as the analysis was not specifically powered to determine potential associations with future outcomes. Future research should explore whether these findings are consistent in a much larger sample of ESUS patients.

As already mentioned, apart from AF, that was the main outcome in our study, we also assessed the recurrence of any ischemic stroke during the follow-up of our ESUS population. In this context, besides the prediction of AF, we also aimed to develop and externally validate an integer-based score for the identification of patients with ESUS at high risk for stroke recurrence. The score was derived and externally validated in 2 large independent multicenter cohorts of consecutive patients with ESUS. The proposed score assigns 1 point per every decade after 35 years of age, 2 points for leukoaraiosis, and 3 points for multiterritorial infarcts. Patients with a score of ≥7 have approximately 3.5 times higher risk of stroke recurrence compared to patients with a score of ≤6.

The association of the 3 measures included in the score with the risk of stroke recurrence is consistent with the results of previous studies: age has been repeatedly identified as a major determinant of stroke recurrence and other stroke outcomes in the overall stroke population, as well as in specific stroke subgroups and in ESUS (273–278).

The presence of multiterritorial infarcts suggests cardiac pathology (e.g., covert atrial fibrillation [AF]) or aortic arch atherosclerosis, both of which confer a significant risk for stroke (73,268,279). Leukoaraiosis was previously shown to be a predictor of stroke recurrence in the non-AF population (280). Although a marker of microangiopathy, leukoaraiosis was also shown to be associated with the probability of AF detection after cryptogenic stroke (190).

It may be argued that the AUC of the proposed score is only moderate (0.63) and therefore insufficiently estimates the risk of stroke recurrence of a specific patient. However, it needs to be emphasized that this (i.e., the estimation of the exact risk of stroke recurrence in a specific patient) is not the question that the present score aims to respond to. Our aim was to develop a score that can identify an ESUS subgroup at high risk for stroke recurrence. This approach is similar to the approach of the CHA2DS2-VASc score, which is recommended for the stratification of stroke risk in patients with AF despite showing a similar AUC in its derivation cohort (0.606) (278): the CHA2DS2-VASc score is not used clinically to provide an accurate estimate of the exact stroke risk of a specific patient, but rather to identify a population at low stroke risk (278). Similarly, the present score identifies an ESUS subgroup (i.e., patients with a score of ≥7) that has approximately 3.5 times higher risk of stroke recurrence compared to patients with lower score.

The proposed score could be potentially useful in the design of future trials of secondary prevention in patients with ESUS, as it could be used to guide eligibility criteria. A similar approach has been used in several randomized controlled trials, which were designed to select a population at higher risk for outcome events (122–124). In addition, the proposed score might potentially be useful for power calculations. In the clinical setting,

the score could be used to assist decisions related to the diagnostic approach: patients at high risk for stroke recurrence could be good candidates for more thorough investigations compared to the set of examinations proposed by the Cryptogenic Stroke/ESUS International Working Group (1). At this stage, we do not suggest the use of the proposed score to guide treatment decisions about secondary prevention.

The strengths of this analysis include the fact that both the derivation and external validation of the score was performed in large independent multicenter cohorts of consecutive patients with ESUS. In addition, the measures of the score can be assessed easily and early after stroke; that is, during hospitalization. Also, the score includes only 3 measures, which makes its utilization easy and straightforward. Once again, limitations include the risk of registration bias within and between the participating registries, the retrospective design of the analysis, and differences in the workup of patients during the inhospital phase. Also, given the retrospective nature of the analysis, the duration of prolonged cardiac monitoring and the degree of leukoaraiosis were not prespecified and available; this may have led to overestimation or underestimation of the risk of stroke recurrence. In addition, as already discussed, for pragmatic reasons, intracranial vascular imaging was not required for the ESUS definition. Still, it is possible that some of our patients with intracranial artery stenosis were erroneously classified as ESUS. Moreover, per the ESUS definition that we used, we did not exclude the presence of complex aortic arch atherosclerosis. Also, we did not take into account genetic, racial, or environmental factors. Our baseline phenotypic data were relatively limited for pragmatic reasons. It would be interesting to assess the subtype of the recurrence of ischemic stroke, but this information is not available.

9 Conclusion

In conclusion, in the context of ambiguity regarding the actual association between ESUS and covert AF as well as regarding the optimal diagnostic workup in patients with ESUS, the present research gathered and analyzed a wide range of clinical, echocardiographic, electrocardiographic and imaging parameters of about 800 well-defined ESUS patients, registered in 3 large stroke registries, in order to identify predictors of covert AF in this stroke population. The result was the derivation of a predictive score, the AF-ESUS score, comprising of 8 covariates (age, hypertension, left atrial dilatation, supraventricular extrasystoles, left ventricular hypertrophy, low LV ejection fraction, subcortical infract and presence of non-stenotic carotid plaques) that have a pathophysiological association with AF and can be easily assessed in daily clinical practice, in the context of the diagnostic evaluation made for the classification of a stroke as ESUS. The proposed score could potentially serve as a tool guiding the selection of ESUS patients for prolonged cardiac monitoring; it may also serve to design future clinical trials aiming to find the appropriate diagnostic and treatment approach in subgroups of this stroke population with specific characteristics. Especially, it could assist the identification of ESUS patients who have low probability of new incident AF as, in our study: among the 42.3% of the population who had an AF-ESUS score of ≤0, only 1.97% had new incident AF during follow-up. The relative risk of new incident AF was 13 times higher in patients with a score of >0 compared to patients with ≤0; the sensitivity and the negative predictive value of a score of ≤0 for new incident AF during follow-up were 94.9% (89.3–98.1%) and 98.1% (95%CI: 95.9–99.1%), respectively. In addition to the high sensitivity and the high

negative predictive value, the developed score has acceptable calibration [AUC 84.8% (95%CI: 79.9– 86.9%)] and discrimination [Hosmer– Lemeshow statistic 4.85 (p=0.77)] and has been validated both internally (with a CV error estimated at 10% (95%CI: 8.6–11.4%)) and externally in an independent cohort of well-defined ESUS patients monitored with ILR. Validating the performance of the AF-ESUS score to predict AF in this ESUS population monitored with ILR, we found that the NPV of the AF-ESUS threshold ≤0 was significantly higher for the identification of ESUS patients with long lasting AF episodes, reaching the value of 100% for episodes lasting >10 h. This finding suggests that in health care settings with limited technical or human resources, ESUS patients with AF-ESUS score >0 may be better candidates for the use of available cardiac rhythm monitoring resources like implantable loop recorders compared with patients with AF-ESUS score ≤0. This personalized strategy of allocation of available resources could increase their diagnostic yield and possibly the associated cost-effectiveness.

In addition to the AF-ESUS score, this research also proposed a simple score, comprising of 3 covariates (age, leukoaraiosis, and multiterritorial infarct) for the identification of ESUS patients at high risk for stroke recurrence. This score could also be potentially useful in the design of future trials of secondary prevention in patients with ESUS, as well as in deciding about the intensity of the diagnostic workup of these patients.

Finally, in the course of collecting and analyzing a wealth of data from a large number of ESUS patients, from this research also emerged an interesting estimation of the prevalence and overlap of potential embolic sources in ESUS. The major overlap of PES, found in our analysis, give a possible explanation to the negative results of the 2 large, randomized trials (NAVIGATE ESUS and RE-SPECT ESUS) and offer a rationale of a

randomized controlled trial of combination of anticoagulation and aspirin for the prevention of stroke recurrence in patients with ESUS.

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11 Appendix 1: Thesis related publications in peer reviewed journals

11.1 Identification of patients with embolic stroke of undetermined source and low risk of new incident atrial fibrillation: The AF-ESUS score

Paper 1.pdf

11.2 Validation of the AF-ESUS score to identify patients with embolic stroke of undetermined source and low risk of device-detected atrial fibrillation

Paper 2.pdf

11.3 Left atrial diameter thresholds and new incident atrial fibrillation in embolic stroke of undetermined source

Paper 3.pdf

11.4 Supraventricular Extrasystoles on Standard 12-lead Electrocardiogram Predict New Incident Atrial Fibrillation after Embolic Stroke of Undetermined Source: The AF-ESUS Study

Paper 4.pdf

11.5 Carotid plaques and detection of atrial fibrillation in embolic stroke of undetermined source

Paper 5.pdf

11.6 Characteristics and outcomes of Embolic Stroke of Undetermined Source according to stroke severity

Paper 6.pdf

11.7 Embolic Stroke of Undetermined Source and Patent Foramen Ovale Risk of Paradoxical Embolism Score Validation and Atrial Fibrillation Prediction

Paper 7.pdf

11.8 Prevalence and Overlap of Potential Embolic Sources in Patients With Embolic Stroke of Undetermined Source

Paper 8.pdf

11.9 Data-driven machine-learning analysis of potential embolic sources in embolic stroke of undetermined source

Paper 9.pdf

11.10 A tool to identify patients with embolic stroke of undetermined source at high recurrence risk

Paper 10.pdf

11.11 External Performance of the HAVOC Score for the Prediction of New Incident Atrial Fibrillation

Paper 11.pdf

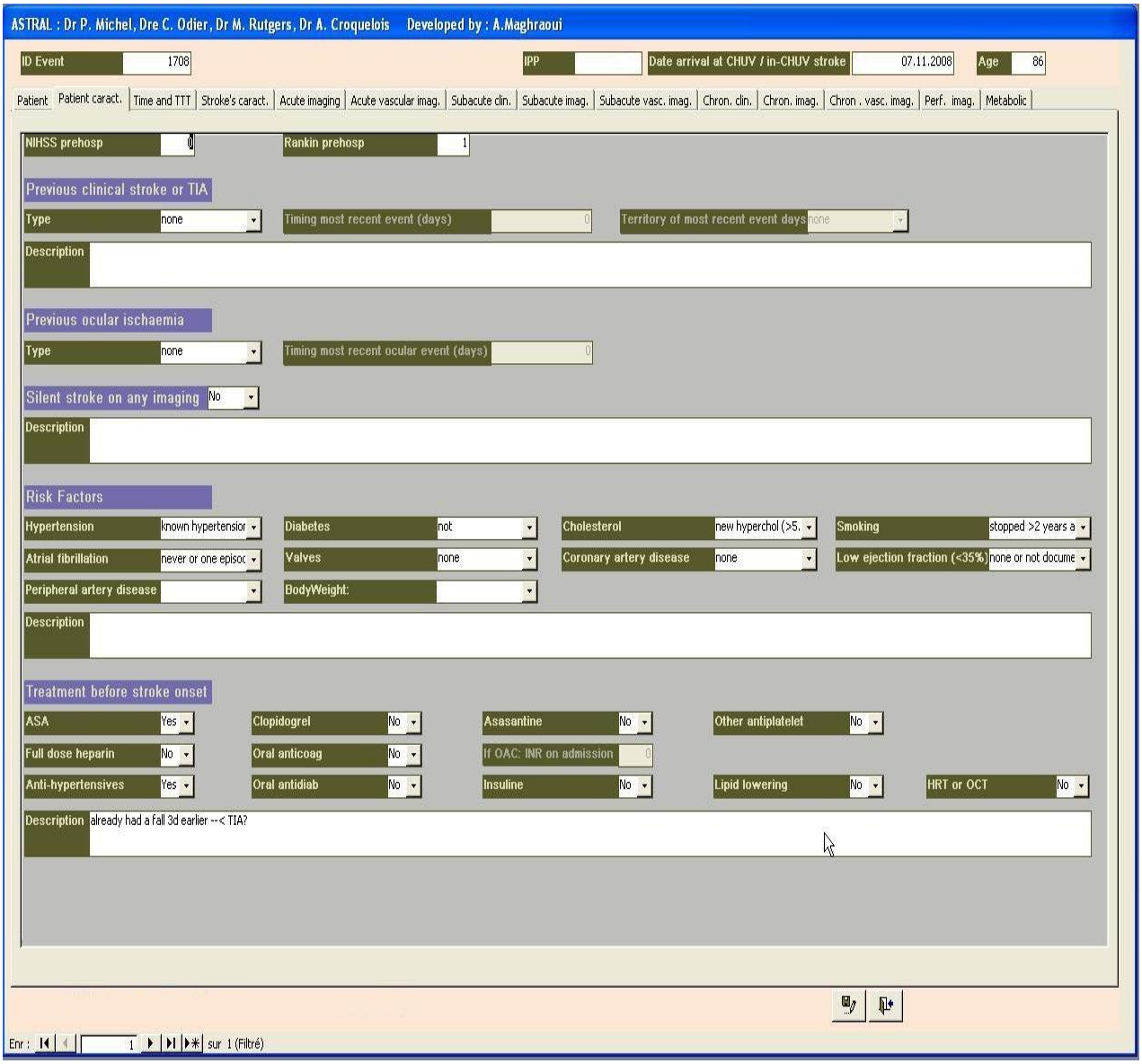
11.12 Atrial Cardiopathy and Likely Pathogenic Patent Foramen Ovale in Embolic Stroke of Undetermined Source

Paper 12.pdf

11.13 Carotid Atherosclerosis and Patent Foramen Ovale in Embolic Stroke of Undetermined Source

Paper 13.pdf

12 Appendix 2: [Interface of the ASTRAL Registry]



13 Appendix 3: [Interface of the LASTRO registry]