DETECTION OF SUBCLINICAL PAROXYSMAL ATRIAL FIBRILLATION AND ITS CORRELATION WITH CANDIDATE GENES IN PATIENTS WITH CRYPTOGENIC ISCHEMIC STROKE AND TIA

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Abstract

Introduction: Cardioembolic etiology is assumed to be the most frequent cause of cryptogenic strokes. The detection of subclinical paroxysmal atrial fibrillation (AF) is important in the correct choice of preventive treatment. The aim of this prospective study was to detect the incidence of AF in patients with a cryptogenic stroke or transient ischemic attack (TIA) and to evaluate the association between the presence of AF and selected single-nucleotide polymorphisms (SNP).

Methods: Patients with a cryptogenic stroke/ TIA (n=100) and a control group (n=15) of volunteers without significant cardiovascular disease were included in the study during the period of 2014 to 2019. To detect AF they underwent 12 months of ECG monitoring using an implanted loop recorder (ILR). Genotyping for SNPs rs10033464, rs2200733, rs225132, and rs2106261 was performed by a high resolution melting analysis.

Results: We found AF to be present in 24 (24%) patients with a cryptogenic stroke/TIA, versus no subjects in the control group. The SNPs rs2106261, rs2200733, rs225132, and rs10033464 were not found to be associated with AF in our study (p=0.240; 1.000; 0.887; 0.589). However, a weak trend for a higher frequency of rs2106261 risk allele A homozygotes was observed in the patients with AF compared to the patients without AF (0.416 vs. 0.263, p=0.073). Homozygotes for allele A of rs2106261 were also present in a significantly higher frequency in AF patients compared to the controls (0.416 vs. 0.133, p = 0.012).

Conclusion: In our study paroxysmal AF was a probable etiological factor in 24% of patients with cryptogenic ischemic stroke / TIA during the 12 months of monitoring. The homozygous allele A of rs2106261 was identified to be the possible genetic risk factor of AF, but this should be verified in larger cohorts.

The study has been registered at www.clinicaltrials.gov, identifier NCT02216370.

Key-words: transient ischemic attack, cryptogenic stroke, atrial fibrillation, implantable loop recorder, single-nucleotide polymorphisms

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INTRODUCTION

An ischemic stroke (IS) is a heterogeneous multi-factor polygenic disease with a high mortality rate and long-term consequences. (1) One of the known, important, and independent risk factors for IS is atrial fibrillation (AF). (2) Its early detection is of fundamental prognostic importance. Paroxysmal AF is often considered a probable cause of cryptogenic stroke. (3) The detection of subclinical paroxysmal AF in this case is difficult because it can be asymptomatic, mainly in older people, and traditional 12-lead or Holter ECGs have a low level of detection due to the limited length of the recording. In recent years the higher detection of AF is due to the introduction of long-term monitoring strategies. Non-stop long-term ECG monitoring using implantable devices is a better and clinically more effective technique than conventional monitoring, in particular following a cryptogenic stroke. In order to achieve maximum AF detection further factors should be identified in order to choose patients for a long-term monitoring. Greater attention is paid to genetic studies under the assumption that genetic tests might help define the subtype of a cryptogenic stroke. (4–8)

The aim of the work was to define the following in a group of patients with a cryptogenic stroke/ transient ischemic attack (TIA): 1) the presence of AF using an implantable ECG loop recorder (ILR); 2) the number of recurrences of stroke/ TIA during the following 12 months; 3) the presence of new ischemic lesions during a magnetic resonance (MRI) examination 12 months later; 4) whether there is a relation between newly-diagnosed AF in our group of Slovak patients and the find of selected single-nucleotide polymorphisms (SNPs) in literature associated with AF and cardioembolic infarction.

METHODS

Characteristics of the groups

One hundred patients with an acute ischemic stroke or TIA were included in the study. The patients were hospitalised within 72 hours from the onset of the ischemic stroke at the Clinic of Neurology of the Faculty Hospital in Nitra from 2014 to 2019 and they met the definition of cryptogenic etiology according to TOAST criteria (inclusion and exclusion criteria) during the screening period. Transient ischemic attack was defined as a temporary neurological deficit of vascular origin without acute ischemic MRI changes. The possible etiological factors of ischemic stroke were excluded in all patients by: CT and MR imaging of the brain and intra/extracranial arteries, hematological testing for thrombophilia, rheumatological tests for vasculitis, 14 days lasting 12-lead Holter ECG for detection of paroxysmal AF, transthoracic and transoesophageal echocardiography for cardioembolism. The control group was made up of 15 volunteers without a significant cardiovascular and cerebrovascular disease adjusted by age and gender. The patients were monitored for 12 months. All patients and volunteers in the control group signed an informed consent form.

Examinations

The diagnosis of an ischemic stroke required consistent neuroimaging findings on CT and MRI. In control individuals MRI had not been performed before participating in the study. All subjects in patient and control groups in addition underwent a follow-up MRI once the monitoring was finished. Standard non-contrast CT and CT angiography of the aortic arch, cervical and intracranial arteries (SIEMENS Somatom Definition, dual source 2x128, Stellar upgrade, SIEMENS Somatom Edge 128), and brain MRI (SIEMENS Skyra 3.0T, SIEMENS Magnetom Avanto 1.5T, SIEMENS Magnetom Symphony 1.5T (protocol T2wTSE SAG + TRA, FLAIR T2wTIRM dark fluid - TRA, DWI - epi2ddiffusion - TRA + ADC, MRA-TOF3D multislabs - TRA + MPR + MIP + VRT, CE imag.-i.v. inject. application + aqua flush 30ml - T1w mpr ISO - TRA + MPR / COR + SAG) were carried out at the JESSENIUS diagnostics centre. The CT and MRI findings were evaluated by independent radiologists.

Implantation of a long-term ECG loop recorder

The implantation of a ILR (REVEAL LINQ / XT, Medtronic Inc., Minneapolis, USA) was carried out within 6 months of the onset of the ischemic stroke/ TIA as arranged with a cardiologist in the following sites: Clinic of Cardiology of the Faculty Hospital in Nitra; Kardiocentrum Nitra s.r.o.; Department of Arrhythmology of the National Institute of Cardiovascular Diseases and the Clinic of Internal Medicine of the Faculty Hospital in Nové Zámky. AF was defined as an episode of irregular cardiac rhythm without the presence of a P wave, lasting for more than 2 minutes. The ECG output obtained from the ILR during check-ups was assessed by an arrhythmologist.

Check-up examination

During the final check-up examination after 12 months we assessed the AF finding, the result of the follow-up brain MRI examination, the recurrence of acute stroke, and a change in treatment in the secondary prevention of acute stroke. In cases where AF was detected by ILR in the course of the 12-month monitoring period the patients and the control subjects underwent an unplanned neurological check-up with a change from antiplatelet treatment to anticoagulation. An unplanned neurological check-up was also undergone in the case of a recurrence of acute stroke.

Genetical analysis

The genetic examination was carried out at the Department of Molecular Biology and Genomics, the Jessenius Faculty of Medicine in Martin and BioMed, Comenius University in Bratislava, in cooperation with the Department of Clinical Biochemistry, Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, and University Hospital Martin. Four SNPs were selected for genetic examination: *rs10033464 (PITX2c gene)*, *rs 2200733 (PITX2c gene)*, *rs225132 (ERRF11 gene)*, and *rs2106261 (ZFHX3 gene)*. The characteristics of the analysed SNPs are shown in Table 1.

Table 1 Selected single-nucleotide polymorphisms (SNP)

SNP	Genetic locus	Chromosome	Major / risk allele	Susceptibility, population
rs2106261	ZFHX3	16q22	G / A	AF, Europeans ≈11% Han Chinese
rs2200733	PITX2c	4q25	C / T	AF, Europeans
rs225132	ERRF11	1	T / G	All types of ischemic stroke, coronary heart disease, Europeans
rs10033464	PITX2c	4q25	G / T	AF, Europeans

Abbreviations: AF – atrial fibrillation

The samples of peripheral blood were taken and dispensed into 3 ml tubes containing 5,4 mg of EDTA (Vacutest Kima S.r.l., Italy). DNA was isolated using DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The concentration and purity of the DNA were determined using Nanodrop (ND-1000, NanoDrop Technologies, Wilmington, DE). All samples were diluted in sterile distilled water to a 25-50 ng/μl final concentration. PCR amplification and genotyping was performed by a High Resolution Melting (HRM) analysis using the Roche LightCycler® 480 Instrument (Roche, USA). The reaction volume consisted of 10 μl of LC480 HRM Master Mix, 2,4 μl of MgCl₂ (25 mM), 2 μl of genomic DNA sample (25–50 ng),

and 0.4 µl of 10µM primers (Ecoli, Czech Republic) filled up to a total volume of 20 µl by redistilled water. The oligonucleotide primers were designed with the online uDesignSM application (www.dna.utah.edu/udesign/index.html). The primer sequences are shown in Table 2. The reaction conditions for rs10033464 and rs2106261 were: 95°C for 10 minutes, (95°C for 10 seconds, 66°C for 15 seconds, 72°C for 10 seconds) x 45 cycles; 72°C for 5 minutes. Heteroduplex PCR was performed at 95°C for 1 minute, 40°C for 1 minute; and c) HRM: 65 to 95°C with a ramp rate set to 0.02°C/second and 25 acquisitions/°C. The reaction conditions for rs225132 and rs 2200733 were: 95°C for 10 minutes, (95°C for 10 seconds, 60°C for 15 seconds, 72°C for 10 seconds) x 45 cycles; 72°C for 5 minutes. Heteroduplex PCR was performed at 95°C for 1 minute, 40°C for 1 minute. HRM analysis was performed at 65 to 95°C with a ramp rate set to 0.02°C/second and 25 acquisitions/°C. To verify the quality of genotype determination several samples from each SNP were bi-directionally sequenced by Sanger sequencing. PCR fragments were purified with ExoSap-IT (Applied Biosystems, Foster City, CA, USA). Sequencing of SNPs was performed using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Ca, USA) on the ABI PRISM 3130x1 automatic sequencer (Applied Biosystems, CA, USA). Sequence data analysis was carried out by using Chromas software (<http://www.technelysium.com.au/chromas.html>).

Table 2 Primers used for HRM

SNP	Primer sequence
rs2106261	F 5' CACAGATAGAGCTCGTCCAGAGAATTG
	R 5' GCCACTTGGATATTTTAATGGATGGTT
rs2200733	F 5' GTGGTACTTGGGTTTTGATTTTGA
	R 5' ACTACCTTAAATATTACCTGTTCTAATTTTCTC
rs225132	F 5' TCAGTGAAGAGGTGGTGAATGTAGGAAT
	R 5' ACCATGTGCCATGAATGCAA
rs10033464	F 5' CAATTTAAATTTTCTTTTTTTTACATTGTTAGAGTCAAGAA
	R 5' CTCAGAGCTTGATGAAAGCACT

Abbreviations: AF – atrial fibrillation

Statistics

We used common methods of descriptive statistics. We evaluated the association between AF and the other categorical variable, SNPs, using a contingency table. The zero hypothesis on the non-existence of an association between atrial fibrillation and another categorical variable (SNP) was tested using a Pearson’s Chi-squared test with Yates’ continuity correction and a Cochran-Armitage trend test. All the data analyses were done in R [15], ver. 3.5.2.

Registration of the study

The study has been registered at www.clinicaltrials.gov under the identifier NCT02216370.

RESULTS

Demographic data

The mean age of the subjects in the patient group was 70.1 years (28–87) vs. 75.4 years (35–90) in the control group. The ratio of women in the patient group was 30% (30) vs. 66.6% (10) in the control group. 90% (90) of patients had experienced a cryptogenic ischemic stroke with a moderate neurological deficit; the median NIHSS was 6.9 (0–15). The ratio of TIA in the patient group was 10% (10). The subjects in the patient group showed an average CHA₂DS₂-VASc score of 3 (2–5) and 1.4 (0–3) in the control group. A total of 68 (68%) of the subjects in the patient group had an arterial hypertension in their personal history (18 (75%) patients with detected AF vs. 9 (60%) of the volunteers in the control group). In the patient group a total of 14 (14%) patients had diabetes mellitus, of which 1 (4.2%) patient had detected AF vs. 2 (13.3%) volunteers in the control group. Clinical characteristics of the involved individuals are summarised in Table 3.

Table 3 Clinical characteristics of involved individuals

Characteristics	With AF (n=24)	Without AF (n=76)	Controls (n=15)
Age (median)	64.29	62.59	75.46
Women % (n)	29.16 (7)	43.42 (23)	66.6 (10)
Cryptogenic stroke	22	68	0
TIA	2	8	0
NIHSS median (range)	6.6 (0–13)	7.1 (0–15)	0
CHA ₂ DS ₂ VASc median (range)	3 (2–4)	3 (2–5)	1.4 (0–3)
Hypertension % (n)	75 (18)	65.8 (50)	60 (9)
Diabetes mellitus % (n)	4.17 (1)	17.11 (13)	13.3 (2)

Abbreviations: TIA – Transient ischemic attack, AF – Atrial fibrillation, NIHSS – The National Institutes of Health Stroke Scale, n – number

Detection of atrial fibrillation, recurrence of acute ischemic stroke, and change of treatment

AF was confirmed in 24% (n = 24) of the patients out of 100 in the patient group. The mean time interval between ILR and AF was 3.9 months (1–11 months). AF was not confirmed in any volunteer in the control group. Within the course of the 12-month monitoring period no subject in the patient nor control groups experienced a clinical or asymptomatic recurrence of an ischemic stroke or a systemic embolism. After the 12 months of monitoring MRI did not reveal any new ischemic brain lesions in the patient and control groups. In the patient group with AF (n = 24) the antiplatelet therapy was changed immediately to anti-coagulants (Table 4).

Table 4 Detection of atrial fibrillation, change in MRI finding, change in preventive treatment after 12 months

<i>Characteristics</i>	<i>Patient group (n = 100)</i>		<i>Control group (n = 15)</i>
	<i>With AF (n=24)</i>	<i>Without AF (n=76)</i>	<i>Without AF (n=15)</i>
<i>Implantation of ICM% (n)</i>	100 (24)	100 (76)	100 (15)
<i>Time before the implantation of ICM (m)</i>	2.5 (1–4)	1–6	1–6
<i>Time preceding detection of AF (m)</i>	3.9 (1–11)	0	0
<i>Recurrence of IS /SE</i>	0	0	0
<i>New MRI ischemic lesions after 12 m % (n)</i>	0 (0)	0 (0)	0 (0)
<i>Change in preventive treatment % (n)</i>	100 (24)	0 (0)	0 (0)

Abbreviations: IS = ischemic stroke, SE = systemic embolisation

3. Analysis of the association of selected SNPs with AF in the patient group

The genotype frequencies of the analysed SNPs in the subgroups of patients and in the controls are shown in Table 5.

SNP *rs2106261* had no statistically significant association with AF ($p = 0.240$). However, a weak trend for a higher frequency of homozygous form of *rs2106261* risk allele A in patients with AF compared to the patients without AF was observed (41.6% vs. 26.3%, $p = 0.073$). Allele G of *rs2106261* was present in 14 (58.4%) patients with AF vs. 56 (73.7%) without AF. The trend for association of *rs2106261* with AF was also found when compared the patients with AF to the controls ($p = 0.058$). The AA homozygotes were 10 patients with AF compared to 2 individuals in the control group and the difference was statistically significant (41.6% vs. 13.3%; $p = 0.012$). Allele G of *rs2106261* was found in 14 (58.4%) patients with AF vs. 13 (86.7%) volunteers in the control group.

We found a weak trend for the association of *rs2106261* with AF ($p = 0.076$). Homozygotes for allele A of *rs2106261* were present in a significantly higher frequency in AF patients compared to the controls (41.6% vs. 13.3%, $p = 0.012$).

A positive finding of *rs2200733* risk allele T homozygotes was observed in 5 (21%) patients with AF vs. 15 (20%) patients without AF. Allele C of *rs2200733* was found in 19 (79%) patients with AF vs. 61 (80%) patients without AF. A statistically significant association of *rs2200733* with AF was not found ($p = 1$). When comparing patients with AF to the control group, homozygotes for risk allele T of *rs2200733* were in 5 (20.8%) patients vs. 3 (20%) volunteers in the control group. Allele T of *rs2200733* in homozygous form was present in 19 (79.8%) patients with AF vs. 12 (80%) volunteers in the control group. In patients with AF compared to the control group, *rs2200733* did not show any statistically significant association with AF ($p = 0.945$).

Homozygotes of *rs225132* minor allele G were 5 (20.8%) patients with AF vs. 19 (25%) patients without AF. Ancestral allele T of *rs225132* was found in 19 (79.2%) patients with AF vs. 57 (75%) patients without AF. A statistically significant association of *rs225132* with AF was not confirmed ($p = 0.887$). When comparing patients with AF to the control group, homozygous allele G of *rs225132* was found in 5 (20.8%) patients vs. 5 (33.3%) volunteers in the control group. Allele T of *rs225132* was confirmed in 19 (79.2%) patients with AF

Table 5 Genotype frequencies of the analysed SNPs in patients and controls

	SNP	rs2106261		rs2200733		rs225132		rs10033464	
		AA	GG + GA	TT	CC + CT	GG	TT + TG	TT	GG + GT
Patients (n = 100)	without AF (n = 76)	0.263 (n = 20)	0.737 (n = 56)	0.200 (n = 15)	0.800 (n = 61)	0.250 (n = 19)	0.750 (n = 57)	0.105 (n = 8)	0.895 (n = 68)
	with AF (n = 24)	0.416 (n = 10)	0.583 (n = 14)	0.208 (n = 5)	0.792 (n = 19)	0.208 (n = 5)	0.792 (n = 19)	0.042 (n = 1)	0.958 (n = 23)
^{2 test} p-value (with vs. without AF)		0.240		1.000		0.887		0.589	
Controls (n = 15)		0.133 (n = 2)	0.867 (n = 13)	0.200 (n = 3)	0.800 (n = 12)	0.333 (n = 5)	0.667 (n = 10)	0.267 (n = 4)	0.733 (n = 11)
	^{2 test} p-value (AF patients vs. controls)	0.058		0.945		0.884		0.261	

vs. 10 (66.4%) healthy volunteers. A statistically significant association of *rs225132* with AF was also not shown when patients with AF were compared to the controls ($p=0.884$).

Homozygous risk allele T of *rs10033464* was present in 1 (4.2%) patient with AF vs. 8 (10.5%) patients without AF. Allele G of *rs10033464* was found in 23 (95.8%) patients with AF vs. 68 (89.4%) patients without AF. A statistically significant association of *rs10033464* with AF was not confirmed ($p=0.589$). When comparing patients with AF to the control group, homozygous *rs10033464* risk allele T was present in 1 (4.2%) patient vs. 4 (26.7%) volunteers in the control group, and the difference was statistically significant ($p=0.047$). Ancestral allele G of *rs10033464* was found in 23 (95.8%) patients vs. 11 (73.3%) volunteers in the control group.

DISCUSSION

In our study we found that paroxysmal AF was a probable etiological factor in 24% of patients with cryptogenic ischemic stroke/ TIA. In the control individuals paroxysmal AF was not present during the 12 months of monitoring. In the randomised control CRYSTAL-AF clinical study the AF detection after 12 months was lower compared to our findings, 12.24% versus 24%. In the above-mentioned study a 30.0% detection rate was only achieved after 36 months of monitoring. (9) In the same manner, a lower detection rate of AF over an average monitoring period of 365 days, compared to our results, was found by Marks et al. (2019) in a retrospective study based on real practice. Using an ILR the authors detected a subclinical AF in 35 patients (19.6%) in a group of 178 patients with cryptogenic ischemic stroke. (10) The detection rate of AF in our patients approximately correlates with the data of the meta-analysis of 28 studies processed by Tsivgoulis et al. (2019) in patients with cryptogenic ischemic stroke and embolic stroke of undetermined source (ESUS) (of which 10 studies monitored patients from 6 to 12 months). (11) The cumulative AF detection rate in patients with ILR was 26% of the above-mentioned meta-analysis. Depending on the duration of monitoring, the authors found significant differences in the AF detection rates ($p < 0.001$) (<6 months: 5%; ≥ 6 and ≤ 12 months: 21%; >12 and <24 months: 26% > 24 months: 34%). They did not confirm any association with other patient characteristics, such as the subtype of ischemic stroke (cryptogenic vs. ESUS), or the time from the beginning of the ischemic stroke to the implantation of the ILR. These findings can be extrapolated to our research. The time of ILR implantation in all our patients within 6 months of the ischemic stroke/ TIA, which was needed for the organisation, performance of examinations and assessment, did not affect the AF detection rate.

In all 24 patients (100%) with AF an antiplatelet therapy was changed to anticoagulation following the recommendations of the ESC (European Society of Cardiology, 2016). (12) We noted no recurrence of ischemic stroke either in the patients or in the control group over the monitored period (12 months), probably due to a short period and an appropriate change of treatment. Unlike our findings, a population study in Oxfordshire, United Kingdom, performed by Li et al. (2015) in patients with a first TIA or cryptogenic ischemic stroke revealed that a 10-year risk of death was 46%, vascular death was 15%, and the recurrence of any form of ischemic stroke was 32%. The risk of recurrence of ischemic stroke/ TIA in this study was lower compared to patients with a cardioembolic etiology ($p = 0.003$). (13) We can only speculate whether the individual efficacy of antiplatelet therapy, possibly depending on p-selectin expression, could be related to a recurrency of ischemic stroke/TIA (14).

A follow-up brain MRI after the 12 months of monitoring did not reveal new asymptomatic ischemic lesions in the patient and control groups. Studies dealing with the MRI detection of new ischemic lesions after a cryptogenic ischemic stroke focus more on proof of low-level symptomatic carotid stenosis, atherosclerosis of small intracranial arteries, or foramen ovale patens as etiological causes. On the other hand, Bal et al. (2012) in a group of 333

(48%) patients with cryptogenic ischemic stroke, out of the total number of patients with a mild stroke/ TIA (National Institutes of Health Stroke Scale score NIHSS ≤ 3), found that 6.6% of patients had new lesions at the 30-day and 14.5% of patients at the 90-day MRI follow-up. (15) This population showed a high rate of silent radiological recurrence, which points to the disease activity. However, the study does not mention the type of therapy administered as part of the secondary prevention of ischemic stroke. In our study the patients had a higher NIHSS score 7, they were given an antiplatelet therapy and an anticoagulation therapy after the finding of AF. The relation between AF and brain lesions caused by a silent cerebral infarction (SCI) at an undefined time, diagnosed by MRI, has been examined in several studies. For example, the Framingham Offspring Study (Das et al., 2008) found a higher risk of SCI in patients with AF (OR 2.16). (16) On the contrary, Gaita et al. (2013) found white matter lesions in 92% of patients with persistent AF, in 89% of patients with paroxysmal AF, and in 46% of patients without AF. A relation between SCI prevalence and the duration of AF was not found. (17)

Regarding the genetic analysis, in the case of rs2106261, we did not confirm a statistically significant association with AF ($p=0.240$). However, we found a slight significant trend towards a higher frequency of rs2106261 AA homozygotes in patients with AF compared to the control individuals ($p=0.076$). Interestingly, a significantly higher frequency of AA homozygotes was also present in patients with AF when compared to the controls ($p=0.012$). Our results can be interpreted in accordance with the published data. Benjamin et al. (2009) in the meta-analysis of GWAS in patients with AF identified of a new susceptibility locus *ZFHX3*, rs2106261 (RR = 1.19; $p = 2.3 \times 10^{-7}$). (4) These findings were replicated in a cohort study of Psaty et al. (2009) from the German AF Network (OR=1.44; $p = 1.6 \times 10^{-11}$; combined RR = 1.25; combined $p = 1.8 \times 10^{-15}$). (5) It is interesting to note that the majority of available publications that identified the association of this SNP variant with AF mainly studied in Asian population by Li et al. (2011) and Zaw et al. (2017). (18, 19) In our study the rs2200733, rs225132, and rs10033464 did not show any significant association with AF. Our findings contradict Henninger et al. (2016) who found that patients with a cardioembolic stroke have a high-risk genetic profile, even despite age and numerous clinical co-morbidities. (20) Pulit et al. (2018) found that a polygenic risk score of AF explains ~20% of the hereditary component of the cardioembolic risk of ischemic stroke. (21) Contrary to our findings, Gretarsdottir et al. (2008) and Gudbjartsson et al. (2009) consider the variant rs2200733 as one of the most important genetic polymorphisms associated with AF and cardioembolic stroke. (6,7) This finding has been consistently replicated and validated in several cohorts of patients with a statistical significance of $p<0.05$, in European and Asian populations by Wnuk et al. (2011), Viviani et al. (2008), Cao et al. (2013), and Sun et al. (2016). (22-25) The published information concerning rs10033464 is inconsistent and our negative findings indirectly and partially match the data in literature. Lemmens et al. (2010) found that rs10033464 is only weakly associated with AF and this variant has demonstrated no relation to ischemic stroke. (26) However, in an isolated population of patients in Iceland, Gretarsdottir et al. (2008) reported a strong association between rs10033464 risk allele T and cardioembolic stroke (OR = 1.27; $p = 6.1 \times 10^{-4}$). (6) A strong association between rs10033464 and AF was also confirmed by Kääb et al. (2009) in cohorts of patients of European origin: the Framingham Heart Study (327 AF patients and 2006 controls, OR = 1.34; 95% CI 1.03–1.75; $p = 0.031$), the German AF Network (1715 patients with AF and 4073 controls, OR = 1.30; 95% CI 1.13–1.51; $p = 0.0002$), and Rotterdam Study (910 AF patients and 5496 controls, OR = 1.17; 95% CI 1.13–1.51; 0.99 – 1.38, $p = 0.07$). (8) On the other hand, the association of rs10033464 with AF was not found in Vanderbilt AF Registry study that was performed in 556 patients with AF and 598 controls without AF (OR = 1.16; 95% CI 0.86–1.56; $p = 0.33$). (8) Interestingly, the autosomal dominant loci in *ankyrin B protein 2* gene on chromosome 4q25 were showed to be associated also with ventricular arrhythmias, particularly long QT syndrome. (27)

In the only publication dealing with *rs225132* in context of AF, Traylor et al. (2012) (METASTROKE Collaboration) identified an association between the *rs225132* variant and AF in patients with ischemic strokes ($p=6.3\times 10^{-8}$ a 5.9×10^{-8}), and this is not in concordance with our findings. (28)

CONCLUSION

In our study we identified the high sensitivity of long-term ECG monitoring using an ILR for detecting subclinical paroxysmal AF as a possible etiological factor of cryptogenic ischemic stroke/TIA comparable to the published sources. In the majority of our patients AF would probably not have been detected at all under a shorter ECG monitoring. The causal relationship between the detected AF and ischemic stroke will require a further analysis. A neurogenic type of AF known as AFDAS (atrial fibrillation detected after stroke), which is triggered by an acute ischemic brain lesion, could explain the time correlation between AF and stroke in some of our patients.

One limitation of the study could be the selection of patients using the TOAST classification that is currently being shown to be insufficient, in particular for defining subgroups of patients with suspected subclinical AF. The selection of patients currently requires a close multidisciplinary (neurology-radiology-cardiology) cooperation, the inclusion of new diagnostic procedures, markers, predictive risk scores that were not known at the time when the methodology for this study was being prepared, or were not routinely carried out. Due to a relatively small number of included patients and control subjects, defining a causal relation between paroxysmal AF detected after ischemic stroke (AFDAS) might appear problematic and will require a further sub-analysis.

For genetic testing we chose four well-known single nucleotide polymorphisms possibly associated with AF - *rs10033464* in *PITX2c* gene, *rs2200733* in *PITX2c* gene, *rs225132* in *ERRF11* gene, and *rs2106261* in *ZFHX3* gene. None of these polymorphisms was shown to be significantly associated with AF in our study. However, in patients with cryptogenic ischemic stroke/ TIA a positive trend for the association of *rs2106261* allele A homozygotes with AF was observed. This insignificant direct correlation may relate to the above-mentioned neurogenic subtype AFDAS which we did not analyse in our study. The possible confounding factor of these findings can be a relatively small cohort of examined individuals, which was due to the financial limitations of a study, especially by the number of available implantable ECG loop recorders. At present, however, 150 candidate genes have been identified for AF. Their variants could lead to a structural transformation of the heart in the form of atrial cardiomyopathy during intra-uterine development, or induced by stress in adulthood. For this reason, to confirm our findings and to clarify the exact mechanisms of involvement of candidate SNPs in AF and cryptogenic ischemic stroke, the future genetic and mainly functional studies including a whole-genome sequencing analysis in larger cohorts of individuals in different populations are necessary. We assume that including a genomic approach in the management of patients with cryptogenic ischemic stroke/ TIA might offer a narrower selection of patients for the implantation of a financially costly ILR and an earlier onset of targeted preventive treatment.

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