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Safety and prognostic relevance of acetylcholine testing in patients with stable myocardial ischaemia or myocardial infarction and non-obstructive coronary arteries

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KEYWORDS

- acute coronary syndrome
- myocardial ischaemia
- spasm

Abstract

Background: Intracoronary provocation testing with acetylcholine (ACh) is crucial for the diagnosis of functional coronary alterations in patients with suspected myocardial ischaemia and non-obstructive coronary arteries.

Aims: Our intention was to assess the safety and predictive value for major adverse cardiovascular and cerebrovascular events (MACCE) in patients presenting with ischaemia with non-obstructive coronary arteries (INOCA) or with myocardial infarction with non-obstructive coronary arteries (MINOCA).

Methods: We prospectively enrolled consecutive INOCA or MINOCA patients undergoing intracoronary ACh provocation testing.

Results: A total of 317 patients were enrolled: 174 (54.9%) with INOCA and 143 (45.1%) with MINOCA. Of these, 185 patients (58.4%) had a positive response to the ACh test. Complications during ACh provocative testing were all mild and transient and occurred in 29 (9.1%) patients, with no difference between patients with positive or negative responses to ACh testing, nor between INOCA and MINOCA patients. A history of paroxysmal atrial fibrillation, moderate/severe diastolic dysfunction and a higher QT dispersion at baseline electrocardiogram were independent predictors of complications. MACCE occurred in 30 patients (9.5%) during a median follow-up of 22 months. The incidence of MACCE was higher among patients with a positive ACh test (24 [13.0%] vs 6 [4.5%], p=0.017), and a positive ACh test was an independent predictor of MACCE.

Conclusions: ACh provocation testing is associated with a low risk of mild and transient complications, with a similar prevalence in both INOCA and MINOCA patients. Importantly, ACh provocation testing can help to identify patients at higher risk of future clinical events, suggesting a net clinical benefit derived from its use in this clinical setting.

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Abbreviations

ACh	acetylcholine
CAD	coronary artery disease
CAG	coronary angiography
CCBs	calcium channel blockers
FFR	fractional flow reserve
INOCA	ischaemia with non-obstructive coronary arteries
LVEF	left ventricle ejection fraction
MACCE	major adverse cardiovascular and cerebrovascular events
MINOCA	myocardial infarction with non-obstructive coronary arteries
SAQ	Seattle Angina Questionnaires
SVT	supraventricular tachycardia
UA	unstable angina
VF	ventricular fibrillation
VT	ventricular tachycardia

Introduction

Coronary vasomotor disorders, both at the microvascular and epicardial level, have been shown to be responsible for myocardial ischaemia in a sizeable group of patients undergoing coronary angiography (CAG). The clinical manifestations range from ischaemia with non-obstructive coronary arteries (INOCA) to myocardial infarction with non-obstructive coronary arteries (MINOCA), as well as life-threatening arrhythmias and sudden cardiac death[1-4]. Intracoronary provocation testing with administration of acetylcholine (ACh) at the time of CAG may elicit epicardial coronary spasm or microvascular spasm in susceptible individuals and, therefore, is of paramount importance in the diagnosis of functional coronary alterations in patients with suspected myocardial ischaemia and non-obstructive coronary artery disease (CAD)[5-7]. However, these studies were mainly focused on patients with INOCA, whilst MINOCA patients were often underrepresented[8,9]. Indeed, intracoronary provocation testing is still underused in clinical practice[10], probably because of concerns about the risk of complications, especially in an acute clinical setting[11]. Therefore, there is a paucity of studies that evaluate both the safety and the prognostic value of this test in a large study population. Consequently, the net clinical benefit deriving from the use of this test in clinical practice remains unknown. Furthermore, there are no studies assessing the safety and the prognostic relevance of this test according to acute (MINOCA) or stable (INOCA) clinical presentations.

Thus, in our study, we aimed to assess the safety of ACh provocation testing, evaluating the prevalence, predictors and the prognostic role of complications that occur during this test across the entire clinical spectrum of patients with myocardial ischaemia and non-obstructive CAD. At the same time, we also aimed to assess the role of intracoronary ACh provocation testing in stratifying the prognosis of these patients.

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Methods

STUDY POPULATION

We prospectively enrolled consecutive patients admitted to the Department of Cardiovascular Sciences of Fondazione Policlinico Universitario A. Gemelli IRCCS in Rome, Italy, undergoing clinically indicated CAG for suspected myocardial ischaemia with angiographic evidence of non-obstructive CAD (angiographically normal coronary arteries or diffuse atherosclerosis with stenosis <50% and/or fractional flow reserve [FFR] >0.80) and undergoing an intracoronary provocation test with ACh from September 2015 to December 2019.

We enrolled patients admitted with both suspected INOCA and MINOCA, diagnosed according to the most recent European Society of Cardiology (ESC) guidelines[5,12,13]. Among patients presenting with suspected MINOCA, we excluded those with obvious causes of myocardial infarction (MI) other than suspected coronary vasomotor abnormalities **(Supplementary Appendix 1)**.

Clinical, laboratory and echocardiographic characteristics of all included patients were collected at admission **(Supplementary Appendix 2, Supplementary Appendix 3)**. The study protocol complied with the Declaration of Helsinki, and the study was approved by our Institutional Review Committee. All patients gave written informed consent to CAG and provocation tests.

CORONARY ANGIOGRAPHY AND INVASIVE PROVOCATION TEST PROTOCOL

CAG was performed through the radial or femoral artery approach. Intracoronary ACh provocation testing was performed immediately after CAG as previously described[4], with a slow rate of manual infusion over a period of 3 minutes, and a "stepwise approach" with a 2-3 minute interval between injections (20-50-100 mcg in the left coronary artery [LCA] and 20-50 mcg in the right coronary artery). The decision to further proceed with testing the LCA with a 200 mcg dose was left to the operator's discretion. CAG was performed 1 minute after each injection and/or when chest pain and/or ischaemic electrocardiogram (ECG) shifts were observed.

The test was considered positive for epicardial coronary spasm in the presence of 1) focal or diffuse epicardial coronary diameter reduction ≥90% in comparison to the relaxed state following intracoronary nitroglycerine administration given to relieve the spasm; 2) reproduction of the patient's symptoms; and 3) ischaemic ECG shifts[14]. Microvascular spasm was diagnosed when typical ischaemic ST-segment changes and angina developed in the absence of epicardial coronary constriction (<90% diameter reduction)[15] **(Supplementary Appendix 4)**[14,15].

ASSESSMENT OF COMPLICATIONS DURING INTRACORONARY PROVOCATION TESTS

Complications occurring during intracoronary provocation testing with ACh were defined as the composite of bradyarrhythmia (asystole or second/third-degree atrioventricular [AV] block lasting more than 3 s), paroxysmal/persistent atrial fibrillation (AF)/supraventricular tachycardia (SVT), ventricular tachycardia (VT),

ventricular fibrillation (VF), and all-cause death. In addition, the individual incidence of each complication was recorded.

CLINICAL OUTCOMES AND FOLLOW-UP

Major adverse cardiovascular and cerebrovascular events (MACCE) were defined as the composite of cardiovascular (CV) death, non-fatal MI, hospitalisation due to unstable angina (UA), and stroke/transient ischaemic attack (TIA). We only counted the first occurrence of MACCE during the follow-up period **(Supplementary Appendix 5)**.

All patients underwent a 24-hour ECG recording after discharge (median 11 months, interquartile range [IQR] 10-12). Occurrence of arrhythmic events was defined as the composite of non-sustained/sustained VT, persistent/permanent AF or any grade of AV block requiring pacemaker implantation. In addition, the individual occurrence of each arrhythmic event was also recorded.

We also recorded any episodes of angina (requiring hospitalisation or not) during the follow-up period and collected the Seattle Angina Questionnaires (SAQ) summary score at 12 months[16]. All patients received clinical follow-ups by telephone interview and/or clinical visits at 6, 12, 24, 36, 48 and 60 months.

STATISTICAL ANALYSIS

Data distribution was assessed according to the Kolgormonov-Smirnov test. Continuous variables were compared using the unpaired Student's t-test or Mann-Whitney U-test, as appropriate, and data were expressed as mean±standard deviation (SD) or as median IQR. Categorical data were evaluated using the χ^2 test or Fisher's exact test as appropriate. A value of $p<0.05$ was considered significant. A multivariable logistic regression analysis for the occurrence of complications during intracoronary positive test was performed including all variables with a p-value<0.05 at univariate analysis. Univariable Cox regression analysis was applied to assess the relation of individual variables with MACCE. Cox regression was then applied to identify variables independently associated with MACCE; to this end, we included in the multivariable model only variables showing $p\leq0.05$ at univariable analysis. All analyses were performed using SPSS version 21 (IBM).

Results

BASELINE CHARACTERISTICS OF STUDY POPULATION

We enrolled 317 patients (mean age 60.5±11.9 years; 177 [55.8%] women) with myocardial ischaemia and non-obstructive coronary arteries undergoing ACh provocation testing. Among them, 174 (54.9%) patients presented with INOCA and 143 (45.1%) with MINOCA.

A positive response to ACh testing was observed in 185 (58.4%). Among the patients with a positive ACh test, 119 (64.3%) developed an epicardial spasm, whereas 66 (35.7%) developed a microvascular spasm. Patients with a positive provocation test, compared to patients with a negative test, had a higher prevalence of grade II or III diastolic dysfunction (29 [15.7%] vs 7 [5.3%], respectively; p=0.004), a lower maximal dose of ACh administered during the test as well as a lower percentage of patients who received a high ACh dose ≥100 mcg (103 [55.7%] vs 92 [69.7%]; p=0.011). Of note, patients with a positive ACh test, compared to those with a negative response, received calcium channel blockers (CCBs) more frequently (174 [94.1%] vs 50 [37.9%], p<0.001) and statins (153 [82.7%] vs 66 [50.0%], p<0.001) as therapy at discharge **(Supplementary Table 1)**.

OCCURRENCE OF COMPLICATIONS DURING ACh INTRACORONARY PROVOCATION TESTING

Complications during ACh testing occurred in 29 (9.1%) patients: 20 (6.3%) patients developed a transient bradyarrhythmia, 8 (2.5%) had paroxysmal/persistent AF/SVT, 1 (0.03%) sustained VT and 1 (0.03%) VF treated with prompt defibrillation. One patient experienced both transient bradyarrhthmias and paroxysmal AF. No deaths were observed.

Patients who developed complications during ACh testing, compared with those who didn't, more frequently had a previous history of paroxysmal AF (11 [37.9%] vs 17 [5.9%], p<0.001) and a higher prevalence of left ventricle (LV) diastolic dysfunction (24 [82.8%] vs 171 [59.4%]; p=0.014), in particular, grade II or III diastolic dysfunction (9 [31.0%] vs 27 [9.4%], p<0.001). Of note, there were no significant differences between the two groups regarding therapy on admission, the maximal dose of ACh administered during the test, or the percentage of patients who received a high ACh dose (≥100 mcg) (all p>0.05). Of interest, there were no differences in the rate of complications between patients with a positive response to ACh testing and patients with a negative response to testing (18 [9.7%] vs 11 [8.3%]; p=0.671), nor between MINOCA and INOCA patients (13 [9.1%] vs 16 [9.2%]; p=0.974) **(Supplementary Figure 1)** or dose of ACh administered (18.4% at 20 mcg, 8.3% at 50 mcg, 7.5% at 100 mcg, 11.1% at 200 mcg; p=0.201) **(Supplementary Figure 2)**.

Moreover, at baseline ECG, patients who developed complications had a higher QT dispersion (40.5 msec [35-53.75] vs 31.0 msec [25-41], p<0.001) compared with those who didn't. Clinical, electrocardiographic, echocardiographic and angiographic features of the overall study population and the occurrence of complications during the provocation test are shown in **Table 1**.

PREDICTORS OF COMPLICATIONS DURING ACh PROVOCATION TESTING

In the univariate logistic regression analysis, a previous history of paroxysmal AF (odds ratio [OR] 9.74, 95% confidence interval [CI]: 3.98-23.86, p<0.001), diastolic dysfunction (OR 3.28, 95% CI: 1.22-8.85; p=0.019), in particular grade II-III diastolic dysfunction (OR 4.35, 95% CI: 1.80-10.50; p=0.001), and QT dispersion at baseline ECG (OR 1.01, 95% CI: 1.00-1.03; p=0.034) were predictors of complications during ACh provocation testing. In the multivariate logistic regression analysis, only a previous history of paroxysmal AF (OR 11.56, 95% CI: 4.32-30.91, p<0.001), grade II-III diastolic dysfunction (OR 3.50, 95% CI: 1.20-10.24; p=0.022), and a higher QT dispersion at baseline ECG (OR 1.02, 95% CI: 1.01-1.03; p=0.021) were independent predictors of complications during intracoronary provocation testing **(Table 2)**.

Table 1. Clinical, ECG, echocardiographic and angiographic features in the overall population and according to the occurrence of complications during ACh provocation test.

Characteristics		Overall population (n=317)	Patients with complications during ACh test (n=29)	Patients without complications during ACh test (n=288)	*p*-value
Clinical characteristics					
Age (mean±SD)		60.5±11.9	56.8±10.6	60.9±12.1	0.061
Male sex, n (%)		140 (44.2)	14 (48.3)	126 (43.8)	0.640
Hypertension, n (%)		209 (65.9)	21 (72.4)	188 (65.3)	0.440
Diabetes, n (%)		63 (19.9)	5 (17.2)	58 (20.1)	0.709
Smoking habit, n, (%)		109 (34.4)	10 (34.5)	99 (34.4)	0.991
Dyslipidaemia, n, (%)		159 (50.2)	13 (44.8)	146 (50.7)	0.547
Obesity, n (%)		25 (7.9)	2 (6.9)	23 (8.0)	1.000
Family history of CAD, n (%)		98 (30.9)	12 (41.4)	86 (29.9)	0.201
Clinical presentation, n (%)	MINOCA, n (%)	143 (45.1)	13 (44.8)	130 (45.1)	0.974
	INOCA, n (%)	174 (54.9)	16 (55.2)	158 (54.9)	
Previous CV history, n (%)		28 (8.8)	4 (13.8)	24 (8.3)	0.323
History of paroxysmal AF, n (%)		28 (8.8)	11 (37.9)	17 (5.9)	<0.001
Laboratory data					
Hb (g/dL), median [IQR]		13.2 [12.4-14.1]	13.3 [12.1-14.9]	13.1 [12.4-14.1]	0.331
WBC ($\times 10^3$/L), median [IQR]		7.0 [6.1-7.9]	6.9 [5.8-8.0]	7.1 [6.1-7.8]	0.824
Serum creatinine on admission (mg/dL), median [IQR]		0.83 [0.71-0.96]	0.78 [0.73-0.98]	0.83 [0.71-0.96]	0.759
Troponin T peak (ng/mL), median [IQR]		0.01 [0.01-0.18]	0.01 [0.01-0.09]	0.01 [0.01-0.18]	0.726
CRP (mg/L), median [IQR]		0.05 [0.05-0.50]	0.05 [0.05-0.50]	0.05 [0.05-0.50]	0.314
Echocardiographic data					
LVEF on admission, %, median [IQR]		61 [58-64]	61 [59-65]	61 [58-64]	0.370
LVEF on admission <50%, n (%)		21 (6.6)	3 (10.3)	18 (6.3)	0.423
Diastolic dysfunction, n (%)		195 (61.5)	24 (82.8)	171 (59.4)	0.014
Grade II or III diastolic dysfunction, n (%)		36 (11.4)	9 (31.0)	27 (9.4)	<0.001
Electrocardiographic data at admission					
QTc interval (msec), median [IQR]		429 [408-455.5]	433 [417.5-466.5]	428.5 [408-455]	0.413
QT dispersion (msec), median [IQR]		32 [25-42]	40.5 [35.0-53.75]	31 [25-41]	<0.001
QRS dispersion (msec), median [IQR]		22 [17-27]	25 [20.5-28.75]	22 [17-27]	0.099
Tp-e interval (msec), median [IQR]		98 [88-110]	94.5 [79-116.5]	98 [88-109.25]	0.519
Tp-e dispersion (msec), median [IQR]		25 [19-32]	24 [17.25-35.25]	25.5 [19-32]	0.718
Tp-e/QTc ratio, median [IQR]		0.23 [0.21-0.26]	0.23 [0.20-0.26]	0.23 [0.21- 0.26]	0.970
Therapy at admission					
Aspirin, n (%)		114 (36.0)	8 (27.6)	106 (36.8)	0.324
Clopidogrel, n (%)		33 (10.4)	6 (20.7)	27 (9.4)	0.057
Ticagrelor, n (%)		6 (1.9)	2 (6.9)	4 (1.4)	0.096
Prasugrel, n (%)		0 (0.0)	0 (0)	0 (0.0)	–
Beta blockers, n (%)		138 (43.5)	13 (44.8)	125 (43.4)	0.883
Calcium channel blockers, n (%)		115 (36.3)	7 (24.1)	108 (37.5)	0.154
ACEi/ARBs, n (%)		193 (60.9)	13 (44.8)	180 (62.5)	0.063
Statins, n (%)		151 (47.6)	9 (31.0)	142 (49.3)	0.060
Diuretics, n (%)		37 (11.7)	5 (17.2)	32 (11.1)	0.327
Nitrates, n (%)		19 (6.0)	2 (6.9)	17 (5.9)	0.688
NOACs, n (%)		29 (9.1)	4 (13.8)	25 (8.7)	0.321
Angiographic data					
Presence of non-obstructive CAD (<50%)		150 (47.3)	10 (34.5)	140 (48.6)	0.146

Table 1. Clinical, ECG, echocardiographic and angiographic features in the overall population and according to the occurrence of complications during ACh provocation test. (cont'd)

Characteristics		Overall population (n=317)	Patients with complications during ACh test (n=29)	Patients without complications during ACh test (n=288)	*p*-value
Provocation test					
Positive, n (%)		185 (58.4)	18 (62.1)	167 (58.0)	0.671
Type of positive response	Epicardial spasm, n (%)	119 (64.3)	10 (55.6)	109 (65.3)	0.414
	Microvascular spasm, n (%)	66 (35.7)	8 (44.4)	58 (34.7)	
High ACh dose (≥100 mcg), n (%)		195 (61.5)	15 (51.7)	180 (62.5)	0.256
ACh maximum dose, median [IQR]		100 [50-100]	100 [35-100]	100 [50-100]	0.105
Complications during provocation test, n (%)					
Bradyarrhythmias		20 (6.3)	20 (69.0)	–	–
AF/SVT		8 (2.5)	8 (27.6)	–	–
VT		1 (0.3)	1 (3.4)	–	–
VF		1 (0.3)	1 (3.4)	–	–
Others		0 (0.0)	0 (0.0)	–	–
Composite of overall complications		29 (9.1)	29 (100.0)	–	–
Therapy at discharge					
Aspirin, n (%)		146 (46.1)	11 (37.9)	135 (46.9)	0.357
Clopidogrel, n (%)		27 (8.5)	5 (17.2)	22 (7.6)	0.077
Ticagrelor, n (%)		5 (1.6)	0 (0.0)	5 (1.7)	1.000
Prasugrel, n (%)		0 (0.0)	0 (0.0)	0 (0.0)	–
Beta blockers, n (%)		96 (30.3)	5 (17.2)	91 (31.6)	0.109
Calcium channel blockers, n (%)		224 (70.7)	22 (75.9)	202 (70.1)	0.519
ACEi/ARBs, n (%)		221 (69.7)	19 (65.5)	202 (70.1)	0.606
Statins, n (%)		219 (69.1)	20 (69.0)	199 (69.1)	0.988
Diuretics, n (%)		37 (11.7)	3 (10.3)	34 (11.8)	1.000
Nitrates, n (%)		7 (2.2)	1 (3.4)	6 (2.1)	0.493
NOACs, n (%)		30 (9.5)	5 (17.2)	25 (8.7)	0.133

ACEi: angiotensin converting enzymes inhibitors; ACh: acetylcholine; AF: atrial fibrillation; ARBs: angiotensin receptor blockers; CAD: coronary artery disease; CRP: C-reactive protein; CV: cardiovascular; Hb: haemoglobin; INOCA: ischaemia with non-obstructive coronary arteries; IQR: interquartile range; LVEF: left ventricle ejection fraction; MINOCA: myocardial infarction with non-obstructive coronary arteries; NOACs: novel oral anticoagulant drugs; SD: standard deviation; SVT: supraventricular tachycardia; VF: ventricular fibrillation; VT: ventricular tachycardia; WBC: white blood count

CLINICAL OUTCOMES ASSOCIATED TO THE OCCURRENCE OF COMPLICATIONS DURING ACh PROVOCATION TESTING

At a median follow-up of 22 months (IQR 13-32 months), MACCE occurred in 30 (9.5%) patients. Of note, there were no differences in the rate of MACCE whether or not there were complications during ACh testing (4 [13.8%] vs 26 [9.0%]; p=0.488, respectively), with no difference in the individual endpoints of CV death, non-fatal MI, hospitalisation for angina or cerebrovascular events **(Supplementary Table 2)**. Moreover, there were no significant differences in the incidence of arrhythmic events detected during the 24-hour ECG recording at 12-month follow-up (6 [20.7%] vs 57 [19.8%]; p=0.695, respectively) as well as for the individual incidence of

Table 2. Predictors of complications during ACh provocative test in the overall population by univariate and multivariate logistic regression analysis.

	Univariate analysis		Multivariable analysis	
	OR (95% CI)	*p*-value	OR (95% CI)	*p*-value
History of paroxysmal AF	9.74 (3.98-23.86)	<0.001	11.56 (4.32-30.91)	<0.001
QT dispersion (msec)	1.01 (1.01-1.03)	0.034	1.02 (1.01-1.03)	0.021
Diastolic dysfunction	3.28 (1.22-8.85)	0.019	2.23 (0.74-6.71)	0.152
Grade II or III diastolic dysfunction	4.35 (1.80-10.50)	0.001	3.50 (1.20-10.24)	0.022

ACh: acetylcholine; AF: atrial fibrillation; CI: confidence interval; OR: odds ratio

non-sustained/sustained VT, permanent AF and any grade AV block requiring pacemaker implantation **(Figure 1)**.

In addition, no significant differences were found between the 2 groups regarding the recurrence of angina (9 [31.0%] vs 63 [21.9%]; p=0.418) and SAQ summary score at 12-month follow-up (84 [IQR 74; 88] vs 82 [IQR 78; 88]; p=0.571) **(Supplementary Table 2)**.

Finally, comparisons of the Kaplan-Meier curves by log-rank test showed no differences between the 2 groups regarding MACCE-free survival (p=0.488) **(Figure 2A)**.

CLINICAL OUTCOMES ACCORDING TO PROVOCATION TEST RESPONSE

The incidence of MACCE was higher among patients with a positive ACh test compared to patients with a negative test (24 [13.0%] vs 6 [4.5%]; p=0.017), mainly driven by a higher rate of hospitalisation for UA (16 [8.6%] vs 4 [3.0%]; p=0.049), without differences in the incidence of CV death (1 [0.5%] vs 0 [0.0%]; p=0.397), non-fatal MI (5 [2.7%] vs 1 [0.8%]; p=0.226) and cerebrovascular events (3 [1.6%] vs 1 [0.8%]; p=0.520). Moreover, patients with a positive ACh provocation test experienced a higher rate of recurrent angina compared to those with a negative response (58 [31.4%] vs 14 [10.6%], p<0.001), and the SAQ summary score at 12-month follow-up was lower in patients with a positive test (82 [IQR 75.5-88] vs 84 [IQR 78-88]; p=0.022) **(Supplementary Table 3, Figure 3)**.

PREDICTORS OF MACCE IN THE OVERALL POPULATION

In the univariate Cox regression analysis, a positive ACh test (hazard ratio [HR] 2.83, 95% CI: 1.16-6.93; p=0.022), MINOCA as clinical presentation (HR 3.23, 95% CI: 1.44-7.26; p=0.004) and left ventricle ejection fraction (LVEF) on admission <50%

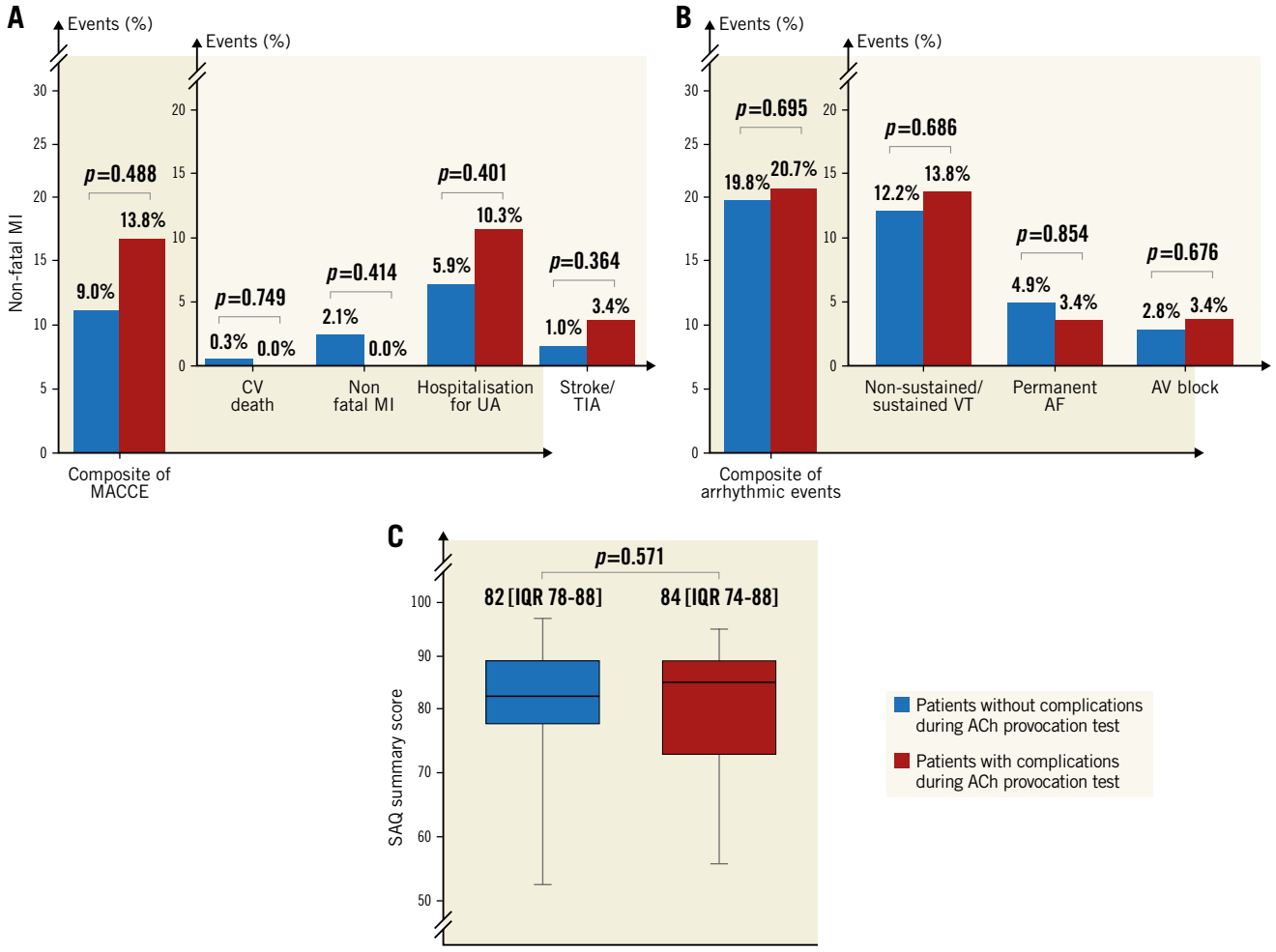


Figure 1. *Clinical outcomes according to the occurrence of complications during ACh provocation test. A) Incidence of composite of MACCE and individual components of MACCE at follow-up according to the occurrence of complications during an ACh provocation test. B) Incidence of composite of arrhythmic events and individual components of arrhythmic events at follow-up according to the occurrence of complications during an ACh provocation test. C) SAQ summary score at follow-up according to the occurrence of complications during an ACh provocation test. ACh: acetylcholine; AF: atrial fibrillation; AV: atrioventricular; CV: cardiovascular; IQR: interquartile range; MACCE: major adverse cardiovascular and cerebrovascular event; MI: myocardial infarction; SAQ: Seattle Angina Questionnaire; TIA: transient ischaemic attack; UA: unstable angina; VT: ventricular tachycardia*

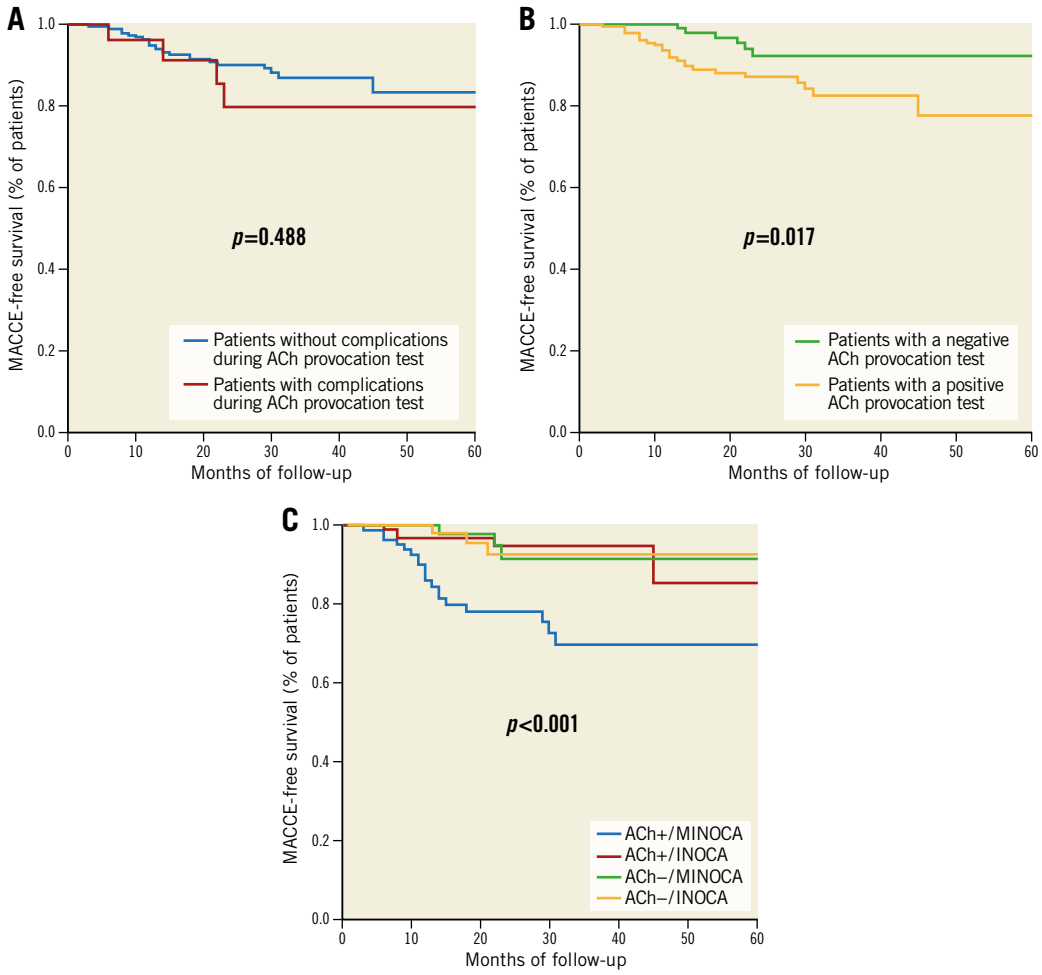


Figure 2. *Survival analysis. A) Survival Kaplan-Meier curve for MACCE according to the occurrence of complications during an ACh provocation test. B) Survival Kaplan-Meier curve for MACCE according to a positive or negative ACh invasive provocation test. C) Survival Kaplan-Meier curve for MACCE according to clinical presentation (MINOCA vs INOCA) and response to provocation test (positive vs negative). Curves are compared by the log-rank test. ACh: acetylcholine; INOCA: ischaemia with non-obstructive coronary arteries; MACCE: major adverse cardiovascular and cerebrovascular event; MINOCA: myocardial infarction with non-obstructive coronary arteries*

(HR 3.18, 95% CI: 1.30-7.81; p=0.011) were the only predictors for the occurrence of MACCE. In multivariate analysis, a positive ACh test (HR 2.82, 95% CI: 1.15-6.93; p=0.023), MINOCA as clinical presentation (HR 3.20, 95% CI: 1.42-7.21; p=0.005) and LVEF on admission <50% (HR 2.60, 95% CI: 1.06-6.39; p=0.037) remained independent predictors for the occurrence of MACCE in the overall population **(Table 3)**. Comparisons of the Kaplan-Meier curves by log-rank test showed that patients with a positive ACh test had a lower MACCE-free survival (p=0.017) **(Figure 2B)**.

CLINICAL OUTCOMES ACCORDING TO PROVOCATION TEST RESPONSE AND CLINICAL PRESENTATION

In a subgroup analysis according to clinical presentation, the incidence of MACCE among patients presenting with MINOCA was higher compared to patients with INOCA (22 [15.4%] vs

Table 3. Predictors of MACCE in the overall population by univariate and multivariate Cox regression analysis.

	Univariate analysis		Multivariable analysis	
	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Positive ACh test	2.83 (1.16-6.93)	0.022	2.82 (1.15-6.93)	0.023
MINOCA as clinical presentation	3.23 (1.44-7.26)	0.004	3.20 (1.42-7.21)	0.005
LVEF on admission <50%	3.18 (1.30-7.81)	0.011	2.60 (1.06-6.39)	0.037

ACh: acetylcholine; CI: confidence interval; HR: hazard ratio; LVEF: left ventricle ejection fraction; MACCE: major adverse cardiovascular and cerebrovascular events; MINOCA: myocardial infarction with non-obstructive coronary arteries

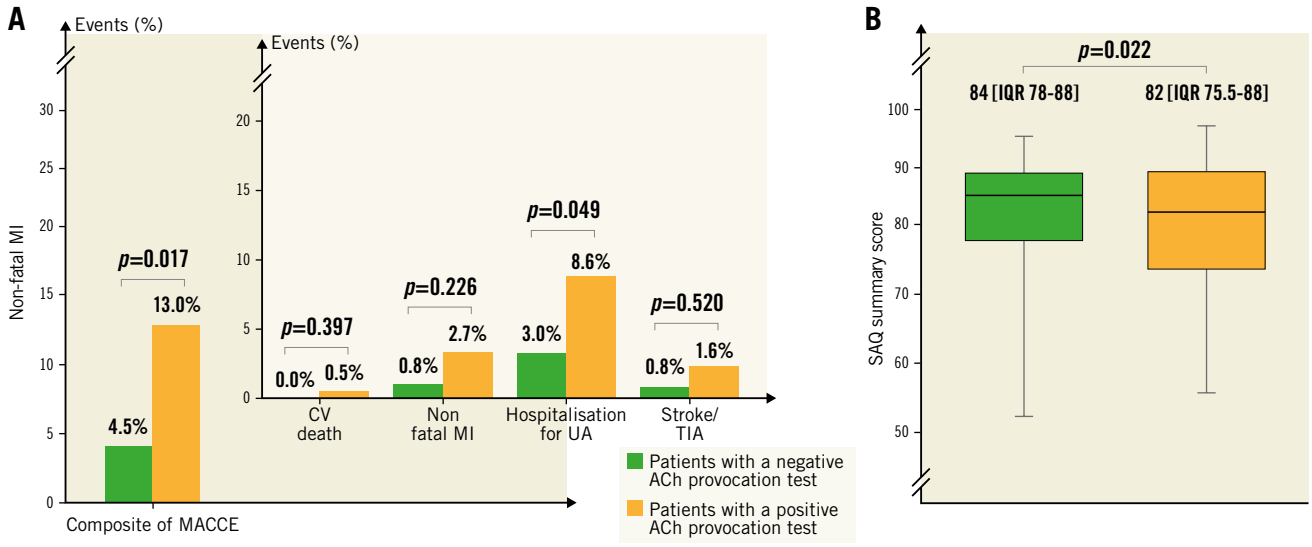


Figure 3. *Clinical outcomes according to provocation test response. A) Incidence of composite of MACCE and individual components of MACCE at follow-up according to a positive or negative ACh invasive provocation test. B) SAQ summary score at follow-up according to a positive or negative ACh invasive provocation test. ACh: acetylcholine; CV: cardiovascular; IQR: interquartile range; MACCE: major adverse cardiovascular and cerebrovascular event; MI: myocardial infarction; SAQ: Seattle Angina Questionnaire; TIA: transient ischaemic attack; UA: unstable angina*

8 [4.6%]; p=0.003), mainly driven by a higher rate of hospitalisation for UA (15 [10.5%] vs 5 [2.9%]; p=0.009). Moreover, patients presenting with MINOCA experienced a higher rate of recurrent angina compared to those with a negative response (43 [30.1%] vs 29 [16.7%]; p=0.018) **(Supplementary Table 4)**.

Furthermore, among MINOCA patients, the incidence of MACCE among patients with a positive ACh test was higher compared to patients with a negative test (19 [22.6%] vs 3 [5.1%]; p=0.006), mainly driven by a higher rate of hospitalisation for UA (13 [15.5%] vs 2 [3.4%]; p=0.021) and with a lower SAQ summary score at follow-up (80 [IQR 74-86] vs 84 [IQR 78-88]; p=0.028) **(Supplementary Table 5)**. On the other hand, among INOCA patients, the incidence of MACCE did not differ between patients with a positive test compared to patients with a negative test (5 [5.0%] vs 3 [4.1%]; p=0.937), as well as the SAQ summary score at follow-up (82 [IQR 77.5-88] vs 84 [IQR 78-90]; p=0.242) **(Supplementary Table 6)**.

In MINOCA patients, in the univariate Cox regression analysis a positive ACh test (HR 4.69, 95% CI: 1.39-15.88; p=0.013) was the only predictor for the occurrence of MACCE **(Supplementary Table 7)**, while in INOCA patients, in the univariate Cox regression analysis an LVEF <50% at admission (HR 9.22, 95% CI: 2.16-39.38; p=0.003) was the only predictor for the occurrence of MACCE **(Supplementary Table 8)**.

Finally, we performed comparisons of the Kaplan-Meier curves by log-rank test according to clinical presentation (MINOCA vs INOCA) and response to provocation testing (positive vs negative), showing that patients with MINOCA and a positive ACh test had the lowest MACCE-free survival, representing the group with the worst prognosis (p<0.001) **(Figure 2C)**.

Discussion

This study represents, to the best of our knowledge, the largest study evaluating both the safety and the prognostic relevance of invasive coronary provocation testing in patients with stable myocardial ischaemia or MI and non-obstructive coronary arteries. It is the first to identify the predictors and the prognostic value of complications occurring during the provocation test and, at the same time, to clarify the role of this test for prognostic stratification in these patients.

The main results of our study can be summarised as follows: 1) the overall rate of complications during ACh provocation testing was rather low (9.1%), with no difference between INOCA or MINOCA patients or between patients with a positive or a negative test response; 2) previous history of paroxysmal AF, moderate-to-severe LV diastolic dysfunction and higher QT dispersion at baseline ECG were the only predictors for the occurrence of complications during the test; 3) the occurrence of complications during intracoronary ACh testing was not associated with a worse prognosis at a medium- to long-term follow-up; 3) a positive ACh test portended worse clinical outcomes, mainly due to a higher rate of rehospitalisation for UA; 4) a positive ACh response predicted a worse prognosis in MINOCA but not INOCA patients.

Our results are in line with previous evidence[9,17-19] reporting low rates of complications in patients undergoing intracoronary provocation testing in experienced centres. We previously showed in 80 MINOCA patients that invasive provocation testing for spasm is safe and identifies a high-risk subset of patients in terms of clinical endpoints and quality of life[4]. Recently Probst et al[9] reported the safety data of ACh provocation testing in 180 patients with myocardial ischaemia and non-obstructive CAD. No irreversible events were observed, and overall transient arrhythmic

complications occurred in 28 (16%) patients, with no differences between INOCA and MINOCA.

Of interest, in our study we did not find any differences in the rate of arrhythmic complications between patients with a positive or negative ACh test, suggesting that the mechanisms that underlie the occurrence of these complications are probably linked to a direct arrhythmogenic effect of ACh rather than being consequent to ACh-induced myocardial ischaemia. Indeed, we demonstrated that the presence of an increased QT dispersion may predispose the development of complications, suggesting that a baseline inhomogeneity of repolarisation may increase the susceptibility to arrhythmias (i.e., a transient exacerbation of QT dispersion due to ACh effect). Accordingly, Suzuki et al[20] demonstrated that patients with vasospastic angina had a higher QT dispersion that was associated with the occurrence of ventricular arrhythmias during provocation tests for spasm, and Kaski et al[21] reported that an increased QT dispersion in patients with vasospastic angina was associated with a higher rate of cardiac arrest and syncope. The non-uniform effects of ACh on ventricular endocardial and epicardial action potential might accentuate QT dispersion[22] and favour the occurrence of provocation-related arrhythmic events, which, however, might also be further favoured by the induction of ischaemia[23].

In addition, a history of paroxysmal AF predicted the occurrence of complications during the test in our patients, in agreement with previous data[24]. Indeed, a previous episode of AF often implies the presence of a latent electrical instability in the atrial myocardium[25-27] which can be triggered by ACh due to its shortening effects on atrial refractory period. Interestingly, the presence of grade II or III LV diastolic dysfunction, which might be related to higher pressure in the left atrium and therefore may enhance the electrical arrhythmogenic effects of ACh, also predicted the occurrence of complications during ACh testing[28,29].

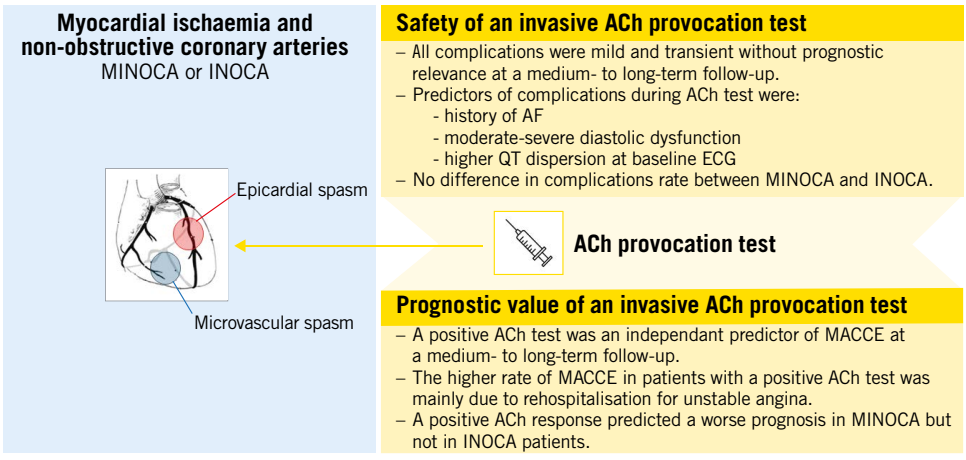
Of importance, we demonstrated that at medium- to long-term follow-up there were no differences in the rate of MACCE between patients experiencing complications compared to those without complications during ACh testing, as well as of arrhythmic events in the 24-hour ECG recording, angina recurrence and the SAQ summary score. Thus, these data can reassure patients and clinicians about the lack of consequences of testing-related events in their clinical outcome. On the other hand, these results should encourage cardiologists to perform provocation testing whenever the presence of a coronary vasomotor disorder is suspected. Indeed, we demonstrated that performing an ACh provocation test has relevant prognostic implications, as patients with a positive test have a higher risk of MACCE at follow-up. Of note, we showed for the first time that the prognostic relevance of a positive provocation test seems to be restricted to MINOCA patients, while for INOCA patients a positive ACh test does not predict a worse clinical outcome **(Central illustration)**. Explanations for these findings are multiple. Indeed, MINOCA patients compared to INOCA have a higher number of events at follow-up, probably due to the more aggressive functional alterations underlying the occurrence of myocardial ischaemia. This point may explain why a provocation test can be more useful in stratifying the prognosis in MINOCA than in INOCA patients. In addition, even though a positive provocation test did not predict the prognosis in INOCA, a tailored therapy was started based on the results of the ACh test, and this may *per se* improve the outcome in these patients[30].

Study limitations

Some limitations of our study should be acknowledged. First, this is a single-centre study. Second, the definition of arrhythmic complications at follow-up has the limit of using a single 24-hour ECG recording, thus likely underestimating the real arrhythmic burden of these patients. Third, we did not invasively measure coronary

EuroIntervention

CENTRAL **ILLUSTRATION** **Safety and prognostic value of an invasive ACh provocation test.**



ACh: acetylcholine; AF: atrial fibrillation; ECG: electrocardiogram; INOCA: ischaemia with non-obstructive coronary arteries; MACCE: major adverse cardiovascular and cerebrovascular event; MINOCA: myocardial infarction with non-obstructive coronary arteries

blood flow and coronary flow reserve and resistance during the invasive study; thus, their potential relationship with the response to vasoconstrictor stimuli remains undetermined. Moreover, we did not assess the presence of endothelial dysfunction at the lowest dose of ACh and its relation to outcomes, potentially confounding the relation between spasm and MACCE. However, MACCE mainly occurred in patients whose ACh test was positive for spasm, while the event rate in patients with an ACh negative response was very low. This suggests that coronary spasm may represent a major determinant for the prognosis of these patients. Fourth, in MINOCA patients taking vasoactive drugs, the provocation tests were not performed after a washout period for CCB and nitrates, potentially interfering with the result of the test. Fifth, the choice to administer the highest dose of ACh for the LCA (200 mcg) was left to the operator's discretion. Finally, the choice to perform a provocation test, especially in patients presenting with stable angina, was left to the operator's discretion. This could have resulted in a selection bias and could explain why the prevalence of MINOCA patients in our study population was higher compared with previous studies.

Conclusions

In conclusion, performing an ACh provocation test in patients with myocardial ischaemia and non-obstructive coronary arteries is safe with a low rate of complications, with no differences between INOCA and MINOCA patients. Moreover, our study identified the presence of paroxysmal AF, moderate/severe diastolic dysfunction, along with QT dispersion, as predictors of complications during intracoronary provocation testing with ACh, helping clinicians to select those patients requiring particular attention during the test. Finally, the demonstration that complications during the ACh test are not associated with a worse prognosis at follow-up may reassure clinicians for post-discharge management. In addition, showing that performing an ACh test can help stratify the prognosis, especially in MINOCA patients, may suggest the presence of a net clinical benefit deriving from its use and supports the decisions of national drug regulatory agencies to approve the use of this test in routine clinical practice.

Impact on daily practice

Performing an acetylcholine provocation test in patients with myocardial ischaemia and non-obstructive coronary arteries is safe with a low rate of complications, with no differences between patients presenting with INOCA or MINOCA. A history of paroxysmal atrial fibrillation, moderate/severe diastolic dysfunction, along with QT dispersion, are predictors of complications during intracoronary provocation testing with acetylcholine, helping clinicians to select patients who require particular attention during the test. Performing an acetylcholine provocation test can help in stratifying the prognosis, especially in MINOCA patients, as patients with a positive test have a higher risk of MACCE at follow-up. These results may suggest the presence of a net clinical benefit deriving from its use.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Supplementary data

Supplementary Appendix 1. Study population.

Supplementary Appendix 2. Echocardiographic assessment.

Supplementary Appendix 3. Electrocardiography.

Supplementary Appendix 4. Coronary angiography and invasive provocative test protocol.

Supplementary Appendix 5. Clinical outcomes and follow-up.

Supplementary Table 1. Clinical, ECG, echocardiographic and angiographic features in the overall population and according to a positive or negative ACh invasive provocative test.

Supplementary Table 2. Clinical outcome and arrhythmic events in the overall population and according to the presence or absence of complications during ACh invasive provocative test.

Supplementary Table 3. Clinical outcome in the overall population and according to a positive or negative ACh invasive provocative test.

Supplementary Table 4. Clinical outcome in the overall population and according to clinical presentation.

Supplementary Table 5. Clinical outcome in the MINOCA population according to a positive or negative ACh invasive provocative test.

Supplementary Table 6. Clinical outcome in the INOCA population according to a positive or negative ACh invasive provocative test.

Supplementary Table 7. Predictors of MACCE in the MINOCA patients by univariate and multivariate Cox regression analysis.

Supplementary Table 8. Predictors of MACCE in the INOCA patients by univariate and multivariate Cox regression analysis.

Supplementary Figure 1. Incidence of complications during acetylcholine provocative test according to (A) a positive or negative acetylcholine invasive provocative test and (B) clinical presentation.

Supplementary Figure 2. Incidence of complications during acetylcholine provocative test according to the dose of acetylcholine administered.

The supplementary data are published online at: https://eurointervention.pcronline.com/doi/10.4244/EIJ-D-21-00971

Supplementary data

Supplementary Appendix 1. Study population

Patients with ischaemia with non-obstructive coronary arteries (INOCA) were defined as those with a stable pattern of typical chest pain on exertion, at rest or both, without any sign of acute myocardial infarction (MI), and/or evidence of inducible myocardial ischaemia undergoing a scheduled hospitalisation for coronary angiography (CAG). Patients with myocardial infarction with non-obstructive coronary arteries (MINOCA) were diagnosed based on clinical evidence of acute myocardial ischaemia, detection of rise and fall of serum troponin T levels with at least one value exceeding the 99th percentile of a normal reference population with an upper limit of 0.014 μg/L and at least one of the following: 1) symptoms of myocardial ischaemia (one or more episodes of chest pain at rest typical enough to suggest a cardiac ischaemic origin in the previous 24 hours); 2) new ischaemic electrocardiogram (ECG) changes (ST-segment and/or T wave abnormalities); 3) development of pathological Q waves; 4) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality in a pattern consistent with an ischaemic aetiology.

Among patients presenting with suspected MINOCA, we excluded those with obvious causes of myocardial infarction (MI) other than suspected coronary vasomotor abnormalities. In particular, we excluded 92 patients with a diagnosis of Takotsubo syndrome confirmed by left ventricle angiography, 54 patients with a suspected diagnosis of myocarditis (diagnosis based on the presence of signs and symptoms of inflammatory activation associated with wall motion abnormalities at left ventricular angiography and echocardiogram suggesting a non-epicardial pattern confirmed by subsequent cardiac magnetic resonance imaging), 130 patients with type 2 MI with mechanism other than suspected vasospasm (e.g., pulmonary embolism, evidence of coronary thrombosis on an unstable plaque confirmed by optical coherence tomography, cardiotoxic drug administration, hypertensive crisis or severe valvulopathies). Moreover, we also excluded 44 patients with permanent atrial fibrillation (AF; defined as any AF that persists despite treatment to restore normal sinus rhythm or that is not treated) and 50 patients with a paced rhythm. Of note, all patients with coronary stenosis ranging from 40% to 50% underwent a fractional flow reserve (FFR) assessment after ACh provocationprovocation test. Per protocol, FFR was performed after ACh test in order to avoid false negative provocationprovocation test results due to intracoronary nitrates administration for FFR. Of note, patients with FFR ≤0.80 were excluded (1 patient). Finally, 317 patients undergoing acetylcholine (ACh) intracoronary provocationprovocation test were included in the analysis.

Supplementary Appendix 2. Echocardiographic assessment

All patients underwent a comprehensive echocardiographic evaluation during hospital admission using a standard ultrasound machine (Artida; Toshiba Medical System) and all images were digitally saved in raw data format to magneto optical discs for offline analysis performed by an experienced echocardiographer. Left ventricle (LV) and left atrial dimensions were obtained by M-mode and two-dimensional (2D) images whereas LV end-diastolic and end-systolic volumes and LV ejection fraction (LVEF) were calculated using the modified Simpson's biplane method.

LV diastolic function was evaluated using transmitral diastolic flow tracing assessed with pulsed-wave Doppler from an apical four-chamber view with E-wave and A-wave velocity measurement. Moreover, also pulsed-wave Tissue Doppler Imaging (TDI) e' velocity (average of lateral and septal basal regions) and average E/e' ratio were assessed [1]. We evaluated four variables for identifying diastolic dysfunction with their abnormal cut-off values: (1) annular e' velocity: septal e' <7 cm/s, lateral e' <10 cm/s; (2) average E/e' ratio >14; (3) left atrial volume index >34 mL/m2; (4) peak tricuspid regurgitation velocity >2.8 m/s. LV diastolic dysfunction was present if more than half of the available parameters met these cut-off values [1]. Diastolic dysfunction was then graded according to the last American Echocardiography guidelines in grade I, II, III.

Supplementary Appendix 3. Electrocardiography

Twelve-leads ECG of the patients were recorded at the time of admission. Surface electrodes were placed to the standard limb and precordial derivations at the resting position, at a rate of 50 mm/s. The QT and QRS duration were measured in all leads. The QT interval was defined as the interval from the onset of the QRS complex to the end of the T and was obtained as the average of all measurable leads. As previously suggested, leads in which the T wave amplitude was very low (i.e., $\leq$0.05 mV [$\leq$0.5 mm]) were excluded. QT dispersion was also calculated as the difference between the maximum and minimum QT intervals recorded. The QT interval was corrected by the heart rate using the Bazett formula: $QTc = QT\sqrt{(R\text{-}R\ interval)}$. A corrected QT dispersion was also calculated from cQT values. The Tp-e interval was defined as the interval from the peak to the end of the T wave in the precordial leads. Tp-e/QT ratios were calculated from these measured values.

Supplementary Appendix 4. Coronary angiography and invasive provocationprovocation test protocol.

When radial approach was chosen, long sheaths were used to prevent radial spasm whereas calcium-channel blockers (CCBs) were avoided. To fully expose all segments of the coronary

arteries, at least two perpendicular projections for right coronary artery (RCA) and four projections for left coronary artery (LCA) were taken. The decision of testing with provocationprovocation test LCA or RCA as first was left to the discretion of the physicians. In INOCA patients taking vasoactive drugs (i.e., CCBs and nitrates), the provocation tests were performed after a wash-out period for these drugs of ≥48 h. A fasting period >12 h was requested in all patients when feasible. In patients with coronary stenosis ranging from 40 to 50%, assessment of FFR, preceded by intracoronary nitroglycerine administration, was performed after the provocationprovocation vasoreactivity test. In patients with MINOCA, the provocationprovocation test was performed during the same procedure of CAG in the acute phase (within 48 hours from admission). Angiographic responses during the provocationprovocation test were assessed in multiple orthogonal views to detect the most severe narrowing and analysed by visual assessment. If either complications and/or a positive response occurred, the test was discontinued, and the higher doses were not administered. All patients with a positive response to provocation ACh testing were discharged from the hospital with an optimal medical treatment, including CCBs and statins up-titrated at the highest tolerated doses.

The test was considered positive for epicardial coronary spasm in the presence of focal or diffuse epicardial coronary diameter reduction ≥90% in comparison with the relaxed state following intracoronary nitroglycerine administration given to relieve the spasm, associated with the reproduction of the patient's symptoms and ischaemic ECG shifts. Microvascular spasm was diagnosed when typical ischaemic ST-segment changes and angina developed in the absence of epicardial coronary constriction (<90% diameter reduction) [15]. Patients who experienced no angina, spasm, or ST-segment shifts were considered to have a negative test response (normal coronary vasoreactivity). Similarly, patients who experienced ischaemic ECG shifts without angina or patients with chest pain without ischaemic ECG shifts were considered to have a negative test response.

Supplementary Appendix 5. Clinical outcomes and follow-up.

Cardiac death included sudden death or death preceded by typical chest pain. Non-fatal MI was defined as typical chest pain at rest associated with ST-segment and/or T-wave abnormalities on the ECG and detection of increased serum troponin T levels. Transient ischaemic attack (TIA) was defined as an acute loss of focal cerebral or ocular function with symptoms lasting less than 24 hours and which after adequate investigation was presumed to be due to embolic or thrombotic vascular disease. Stroke was defined as a neurological deficit attributed to an acute focal injury of

the central nervous system by a vascular cause, including cerebral infarction, intracerebral haemorrhage and subarachnoid haemorrhage.

Supplementary Table 1. Clinical, ECG, echocardiographic and angiographic features in the overall population and according to a positive or negative ACh invasive provocation test.

Characteristics	Overall population (n=317)	Patients with a positive ACh test (n=185)	Patients with a negative ACh test (n=132)	p-value
Clinical characteristics				
Age (mean±standard deviation)	60.5±11.9	61.3±11.6	59.3±12.1	0.137
Male sex, n (%)	140 (44.2)	78 (42.2)	62 (47.0)	0.395
Hypertension, n (%)	209 (65.9)	119 (64.3)	90 (68.2)	0.475
Diabetes, n (%)	63 (19.9)	35 (18.9)	28 (21.2)	0.614
Smoking habit, n (%)	109 (34.4)	62 (33.5)	47 (35.6)	0.699
Dyslipidaemia, n (%)	159 (50.2)	87 (47.0)	72 (54.5)	0.187
Obesity, n (%)	25 (7.9)	16 (8.6)	9 (6.8)	0.551
Family history of CAD, n (%)	98 (30.9)	53 (28.6)	45 (34.1)	0.301
Clinical presentation, n (%)				0.901
MINOCA, n (%)	143 (45.1)	84 (45.4)	59 (44.7)	
INOCA, n (%)	174 (54.9)	101 (54.6)	73 (55.3)	
Previous CV history, n (%)	28 (8.8)	14 (7.6)	14 (10.6)	0.347
History of paroxysmal AF, n (%)	28 (8.8)	15 (8.1)	13 (9.8)	0.590
Laboratory data				
Hb (g/dL), median [IQR]	13.2 [12.4-14.1]	13.2 [12.4-14.2]	13.1 [12.2-14.1]	0.250
WBC (x10^3/L), median [IQR]	7.0 [6.1-7.9]	6.8 [5.9-7.9]	7.1 [6.2-7.9]	0.264
Serum creatinine on admission (mg/dL), median [IQR]	0.83 [0.71-0.96]	0.83 [0.71-0.94]	0.81 [0.70-1.0]	0.469
Troponin T peak (ng/mL), median [IQR]	0.01 [0.01-0.18]	0.01 [0.01-0.15]	0.01 [0.01-0.25]	0.330
CRP (mg/L), median [IQR]	0.05 [0.05-0.50]	0.05 [0.05-0.50]	0.05 [0.05-2.38]	0.148

Echocardiographic data				
LVEF on admission, % median [IQR]	61 [58-64]	61 [58-64]	61 [58-64]	0.594
LVEF on admission <50% n, (%)	21 (6.6)	15 (8.1)	6 (4.5)	0.209
Diastolic dysfunction, n (%)	195 (61.5)	119 (64.3)	76 (57.6)	0.223
Grade II or III diastolic dysfunction, n (%)	36 (11.4)	29 (15.7)	7 (5.3)	**0.004**
Electrocardiographic data at admission				
QTc interval (msec), median [IQR]	429 [408-455.5]	430 [409-460.25]	429 [403-452]	0.511
QT dispersion (msec), median [IQR]	32 [25-42]	32.5 [27-42]	32 [24-43]	0.488
QRS dispersion (msec), median [IQR]	22 [17-27]	21.5 [17-28]	23 [18-26.25]	0.592
Tp-e interval (msec), median [IQR]	98 [88-110]	98 [88-110.75]	97 [88-108.25]	0.796
Tp-e dispersion (msec), median [IQR]	25 [19-32]	26 [19-32.75]	25 [19-32]	0.747
Tp-e/QTc ratio, median [IQR]	0.23 [0.21-0.26]	0.23 [0.21-0.26]	0.22 [0.21-0.27]	0.876
Therapy at admission				
Aspirin, n (%)	114 (36.0)	69 (37.3)	45 (34.1)	0.558
Clopidogrel, n (%)	33 (10.4)	21 (11.4)	12 (9.1)	0.516
Ticagrelor, n (%)	6 (1.9)	5 (2.7)	1 (0.8)	0.407
Prasugrel n (%)	0 (0.0)	0 (0)	0 (0.0)	-
Beta blockers, n (%)	138 (43.5)	79 (42.7)	59 (44.7)	0.724
Calcium-channel blockers, n (%)	115 (36.3)	69 (37.3)	46 (34.8)	0.655
ACEi/ARBs, n (%)	193 (60.9)	112 (60.5)	81 (61.4)	0.882
Statins, n (%)	151 (47.6)	97 (52.4)	54 (40.9)	0.043
Diuretics, n (%)	37 (11.7)	23 (12.4)	14 (10.6)	0.618
Nitrates, n (%)	19 (6.0)	13 (7.0)	6 (4.5)	0.359
NOACs, n (%)	29 (9.1)	18 (9.7)	11 (8.3)	0.671

Angiographic data				
Presence of non-obstructive CAD (<50%)	150 (47.3)	91 (49.2)	59 (44.7)	0.430
Provocation test				
High ACh dose (≥100 mcg), n (%)	195 (61.5)	103 (55.7)	92 (69.7)	**0.011**
ACh maximum dose median [IQR]	100 [50-100]	100 [50-100]	100 [50-100]	**0.007**
Complications during provocation test, *n, (%)*				
Bradyarrhythmias	20 (6.3)	11 (5.9)	9 (6.8)	0.753
AF/SVT	8 (2.5)	6 (3.2)	2 (1.5)	0.334
VT	1 (0.3)	1 (0.5)	0 (0.0)	0.398
VF	1 (0.3)	1 (0.5)	0 (0.0)	0.398
Others	0 (0.0)	0 (0.0)	0 (0.0)	-
Composite of overall complications	29 (9.1)	18 (9.7)	11 (8.3)	0.671
Therapy at discharge				
Aspirin, n (%)	146 (46.1)	87 (47.0)	59 (44.7)	0.682
Clopidogrel, n (%)	27 (8.5)	17 (9.2)	10 (7.6)	0.612
Ticagrelor, n (%)	5 (1.6)	4 (2.2)	1 (0.8)	0.406
Prasugrel, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	-
Beta blockers, n (%)	96 (30.3)	55 (29.7)	41 (31.1)	0.799
Calcium-channel blockers, n (%)	224 (70.7)	174 (94.1)	50 (37.9)	**<0.001**
ACEi/ARBs, n (%)	221 (69.7)	128 (69.2)	93 (70.5)	0.809
Statins, n (%)	219 (69.1)	153 (82.7)	66 (50.0)	**<0.001**
Diuretics, n (%)	37 (11.7)	27 (14.6)	10 (7.6)	0.055
Nitrates, n (%)	7 (2.2)	4 (2.2)	3 (2.3)	0.947
NOACs, n (%)	30 (9.5)	19 (10.3)	11 (8.3)	0.561

ACEi: angiotensin converting enzymes inhibitors; ACh: acetylcholine; AF: atrial fibrillation; ARBs: angiotensin receptor blockers; CAD: coronary artery disease; CRP: C-reactive protein; CV: cardiovascular; Hb: haemoglobin; INOCA: ischaemia with non-obstructive coronary arteries; IQR: interquartile range; LVEF: left ventricle ejection fraction; MINOCA: myocardial infarction with

non-obstructive coronary arteries; NOACs: novel oral anticoagulant drugs; SVT: supraventricular tachycardia; VF: ventricular fibrillation; VT: ventricular tachycardia; WBC: white blood count

Supplementary Table 2. Clinical outcome and arrhythmic events in the overall population and according to the presence or absence of complications during ACh invasive provocation test.

Characteristics	Overall population (n=317)	Presence of complications (n=29)	Absence of complications (n=288)	p-value
MACCE, n (%)	30 (9.5)	4 (13.8)	26 (9.0)	0.488
CV death, n (%)	1 (0.3)	0 (0.0)	1 (0.3)	0.749
Non-fatal MI, n (%)	6 (1.9)	0 (0.0)	6 (2.1)	0.414
Hospitalisation for UA, n (%)	20 (6.3)	3 (10.3)	17 (5.9)	0.401
Stroke/TIA, n (%)	4 (1.3)	1 (3.4)	3 (1.0)	0.364
Recurrent angina, n (%)	72 (22.7)	9 (31.0)	63 (21.9)	0.418
SAQ summary score, median [IQR]	82 [77.5-88]	84 [74-88]	82 [78-88]	0.571
24-hour ECG recording time from dismission months, median [IQR]	11 [10-12]	11 [9-12]	11 [10-12]	0.211
Incidence of arrhythmic events, n (%)	63 (19.9)	6 (20.7)	57 (19.8)	0.695
Non-sustained/sustained VT n, (%)	39 (12.3)	4 (13.8)	35 (12.2)	0.686
Permanent AF, n (%)	15 (4.7)	1 (3.4)	14 (4.9)	0.854
Any grade AV block requiring pacemaker implantation, n (%)	9 (2.8)	1 (3.4)	8 (2.8)	0.676
Follow-up time months, median [IQR]	22 [13-32]	26 [12.5-38.5]	22 [13-32]	0.482

ACh: acetylcholine; AF: atrial fibrillation; AV: atrio ventricular; CV: cardiovascular; ECG: electrocardiogram; IQR: interquartile range; MACCE: major adverse cardiovascular and cerebrovascular events; MI: myocardial infarction; SAQ: Seattle Angina Questionnaire; TIA: transient ischaemic attack; UA: unstable angina; VT: ventricular tachycardia

Supplementary Table 3. Clinical outcome in the overall population and according to a positive or negative ACh invasive provocation test.

Characteristics	Overall population (n=317)	Positive ACh provocation test (n=185)	Negative ACh provocation test (n=132)	p-value
MACCE, n (%)	30 (9.5)	24 (13.0)	6 (4.5)	**0.017**
CV death, n (%)	1 (0.3)	1 (0.5)	0 (0.0)	0.397
Non-fatal MI, n (%)	6 (1.9)	5 (2.7)	1 (0.8)	0.226
Hospitalisation for UA, n (%)	20 (6.3)	16 (8.6)	4 (3.0)	**0.049**
Stroke/TIA, n (%)	4 (0.8)	3 (1.6)	1 (0.8)	0.520
Recurrent angina, n (%)	72 (22.7)	58 (31.4)	14 (10.6)	**<0.001**
SAQ summary score, median [IQR]	82 [77.5-88]	82 [75.5-88]	84 [78-88]	**0.022**
Follow-up time months, median [IQR]	22 [13-32]	22 [13-32]	22 [12.25-32]	0.670

ACh: acetylcholine; CV: cardiovascular; IQR: interquartile range; MACCE: major adverse cardiovascular and cerebrovascular events; MI: myocardial infarction; SAQ: Seattle Angina Questionnaire; TIA: transient ischaemic attack; UA: unstable angina

Supplementary Table 4. Clinical outcome in the overall population and according to clinical presentation.

Characteristics	Overall population (n=317)	MINOCA (n=143)	INOCA (n=174)	p-value
MACCE, n (%)	30 (9.5)	22 (15.4)	8 (4.6)	**0.003**
CV death, n (%)	1 (0.3)	1 (0.7)	0 (0.0)	0.279
Non-fatal MI, n (%)	6 (1.9)	3 (2.1)	3 (1.7)	0.833
Hospitalisation for UA, n (%)	20 (6.3)	15 (10.5)	5 (2.9)	**0.009**
Stroke/TIA, n (%)	4 (0.8)	3 (2.1)	1 (0.6)	0.250
Recurrent angina, n (%)	72 (22.7)	43 (30.1)	29 (16.7)	**0.018**
SAQ summary score, median [IQR]	82 [77.5-88]	80 [76-88]	84 [78-88.5]	0.077
Follow-up time months, median [IQR]	22 [13-32]	23 [14-34]	21 [12-32]	0.199

CV: cardiovascular; INOCA: ischaemia with non-obstructive coronary arteries; IQR: interquartile range; MACCE: major adverse cardiovascular and cerebrovascular events; MI: myocardial infarction; MINOCA: myocardial infarction with non-obstructive coronary arteries; SAQ: Seattle Angina Questionnaire; TIA: transient ischaemic attack; UA: unstable angina

Supplementary Table 5. Clinical outcome in the MINOCA population according to a positive or negative ACh invasive provocation test.

Characteristics	**MINOCA population** (n=143)	**Positive ACh provocation test** (n=84)	**Negative ACh provocation test** (n=59)	**p-value**
MACCE, n, (%)	22 (15.4)	19 (22.6)	3 (5.1)	**0.006**
CV death, n (%)	1 (0.7)	1 (1.2)	0 (0.0)	0.375
Non-fatal MI, n (%)	3 (2.1)	3 (3.6)	0 (0.0)	0.148
Hospitalisation for UA, n (%)	15 (10.5)	13 (15.5)	2 (3.4)	**0.021**
Stroke/TIA, n (%)	3 (2.1)	2 (2.4)	1 (1.7)	0.764
Recurrent angina, n (%)	43 (30.1)	37 (44.0)	6 (10.1)	**<0.001**
SAQ summary score, median [IQR]	80 [76-88]	80 [74-86]	84 [78-88]	**0.028**
Follow-up time months, median [IQR]	23 [14-34]	22.5 [14-33.75]	24 [14-35]	0.757

ACh: acetylcholine; CV: cardiovascular; IQR: interquartile range; MACCE: major adverse cardiovascular and cerebrovascular events; MI: myocardial infarction; MINOCA: myocardial infarction with non-obstructive coronary arteries; TIA: transient ischaemic attack; SAQ: Seattle Angina Questionnaire; UA: unstable angina

Supplementary Table 6. Clinical outcome in the INOCA population according to a positive or negative ACh invasive provocation test.

Characteristics	INOCA population (n=174)	Positive ACh provocation test (n=101)	Negative ACh provocation test (n=73)	p-value
MACCE, n (%)	8 (4.6)	5 (5.0)	3 (4.1)	0.937
CV death, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	-
Non-fatal MI, n (%)	3 (1.7)	2 (2.0)	1 (1.4)	0.878
Hospitalisation for UA, n (%)	5 (2.9)	3 (3.0)	2 (2.7)	0.986
Stroke/TIA, n (%)	1 (0.6)	1 (1.0)	0 (0.0)	0.480
Recurrent angina, n (%)	29 (16.7)	21 (20.8)	8 (11.0)	0.207
SAQ summary score, median [IQR]	84 [78-88.5]	82 [77.5-88]	84 [78-90]	0.242
Follow-up time months, median [IQR]	21 [12-32]	22 [13-32]	20 [12-31.5]	0.438

ACh: acetylcholine; CV: cardiovascular; INOCA: ischaemia with non-obstructive coronary arteries; IQR: interquartile range; MACCE: major adverse cardiovascular and cerebrovascular events; MI: myocardial infarction; SAQ: Seattle Angina Questionnaire; TIA: transient ischaemic attack; UA: unstable angina

Supplementary Table 7. Predictors of MACCE in the MINOCA patients by univariate and multivariable Cox regression analysis.

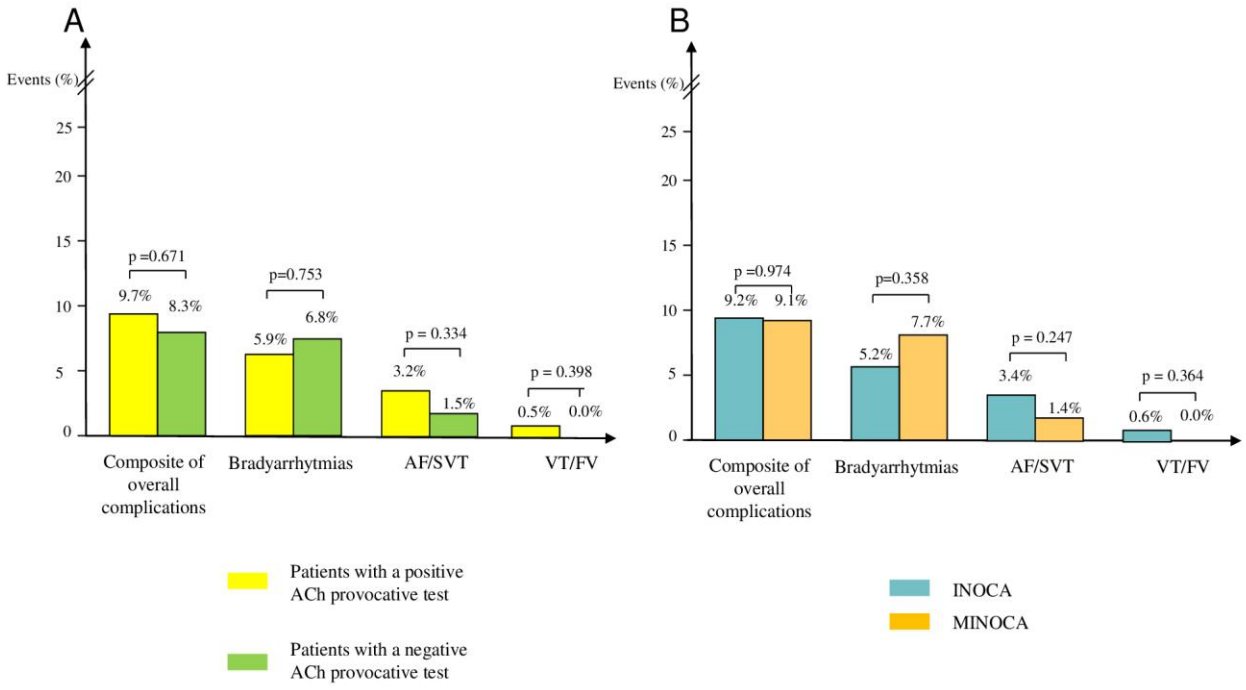
	Univariate analysis		Multivariable analysis	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
Positive ACh test	4.69 (1.39-15.88)	**0.013**	-	-

ACh: acetylcholine; CI: confidence interval; HR: hazard ratio; MACCE: major adverse cardiovascular and cerebrovascular events; MINOCA: myocardial infarction with non-obstructive coronary arteries

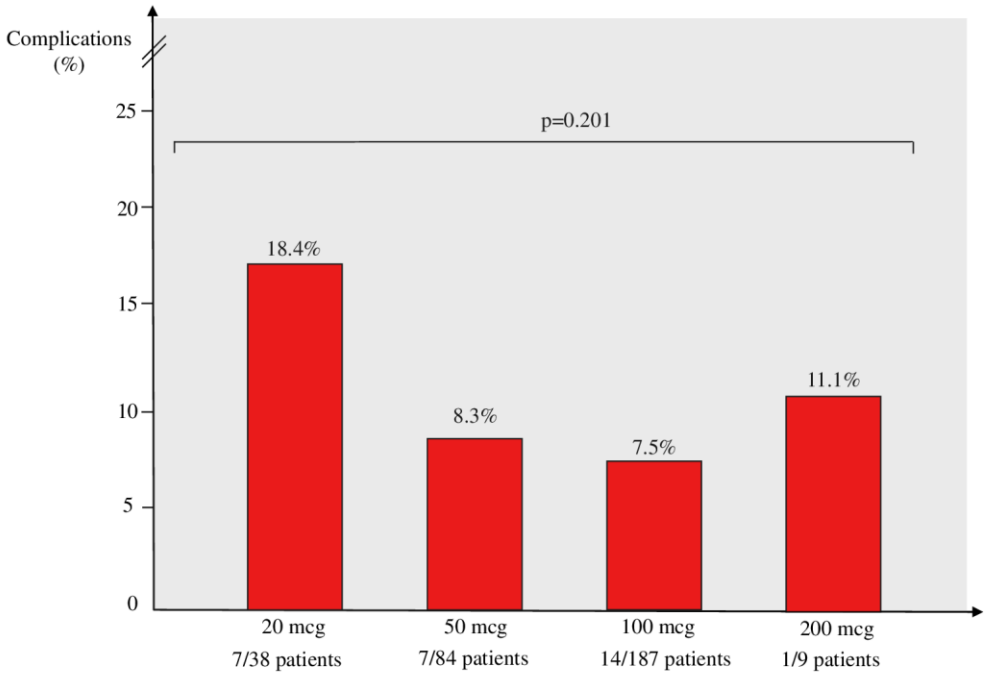
Supplementary Table 8. Predictors of MACCE in the INOCA patients by univariate and multivariable Cox regression analysis.

	Univariate analysis		Multivariable analysis	
	HR (95% CI)	**p-value**	**HR (95% CI)**	**p-value**
LVEF on admission <50%	9.22 (2.16-39.38)	**0.003**	-	-

CI: confidence interval; HR: hazard ratio; INOCA: myocardial ischaemia with non-obstructive coronary arteries; LVEF: left ventricle ejection fraction; MACCE: major adverse cardiovascular and cerebrovascular events



Supplementary Figure 1. Incidence of complications during acetylcholine provocation test according to (A) a positive or negative acetylcholine invasive provocation test and (B) clinical presentation. ACh: acetylcholine; AF: atrial fibrillation; INOCA: ischaemia with non-obstructive coronary arteries; MINOCA: myocardial infarction with non-obstructive coronary arteries; SVT: supraventricular tachycardia; VF: ventricular fibrillation; VT: ventricular tachycardia



Supplementary Figure 2. Incidence of complications during acetylcholine provocation test according to the dose of acetylcholine administered.