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The prognostic value of nitrotyrosine levels in coronary heart disease: long-term evaluation in the Acute Coronary Syndrome Registry Strategy (ERICO study)

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ABSTRACT

Introduction: We aimed to analyze the association of nitrotyrosine (N-TYR) levels and long-term survival in an ongoing coronary heart disease (CHD) prospective cohort, the Acute Coronary Syndrome Registry Strategy (ERICO study).

Methods: N-TYR levels collected during acute and subacute phase from onset of acute coronary syndrome (ACS) symptoms (myocardial infarction and unstable angina) were evaluated in 342 patients. We calculated case-fatality rates (180-days, 1 year, 2 years and 4 years) and survival analyses up to 4 years using Kaplan-Meier curves and Cox regression with respective cumulative hazard ratios (95% confidence interval; 95%CI), according to N-TYR tertiles up to 4 years of follow-up. Models are presented as crude, age and sex-adjusted and further adjusted for lipids and other confounders.

Results: Overall, median level of N-TYR was 208.33 nmol/l (range: 3.09 to 1500 nmol/l), regardless ACS subtype. During follow-up of 4 years, we observed 44 (12.9%) deaths. Overall survival rate was 298 (87.1%) (Survival days: 1353, 95%CI: 1320–1387 days). N-TYR levels did not associate with mortality / survival rates up to 4 years.

Conclusions: No relationship was found between N-TYR levels and mortality rates after ACS during 4-year follow-up in the ERICO study.

1. Introduction

Globally, cardiovascular disease (CVD) is one of the comorbidities responsible for the higher rates of mortality (17.6 million deaths) and years of life lost (YLLs) [1]. In Brazil, CVD is also the main cause of death since the 1960s, and coronary heart disease (CHD) accounted for 31% of CVD mortality [2]. Indeed, mortality after acute coronary syndrome (ACS), which comprises unstable angina (UA) and myocardial infarction (MI) with or without ST-segment elevation, is implicated with high morbidity and mortality rates worldwide, particularly in low-middle income countries [3,4].

In this context, biomarkers as determinants of prognosis after ACS might have an important role in predicting cardiovascular risk due their participation in the atherosclerotic process [5–7]. There is an increasing evidence that the presence of elevated levels of nitrotyrosine (N-TYR) may play a crucial role in determining tissue injury in atherosclerosis [8–12]. Specifically, N-TYR, which is a potential oxidative stress biomarker associated to atherosclerotic plaque nitration and tissue damage, has a potential role as inflammatory mediator in coronary artery disease (CAD) and may have implications for atherosclerosis risk assessment [8]. Further, nitrotyrosine was found in complex heterogeneous cellular plaques and myointimal plaques, which is also correlated with plaque instability in patients and promote atherogenesis [13,14].

Despite the importance of the oxidation and inflammatory process involving N-TYR after ACS, few have investigated the influence of N-

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TYR levels on acute coronary syndrome mortality [15].

Therefore, we aimed to evaluate the long-term prognostic value of N-TYR in ACS regarding to all-cause, CVD and CHD mortality rates among participants from the Acute Coronary Syndrome Registry Strategy (ERICO study), a community-based cohort conducted in a low-income area in the city of Sao Paulo, Brazil.

2. Methods

2.1. Study design and sample

This prospective study evaluated in a subsample of 342 ACS participants from the ERICO study, who had their N-TYR levels quantified from 24 h to 10 days of the onset of ACS symptoms. In brief, ERICO study, which is an ongoing cohort, was launched in February 2009 and included its last participant in December 2013, in the Hospital Universitário from University of São Paulo (HU-USP) [16]. The HU-USP is a 260-bed teaching community hospital in the borough of Butantã that had a population of 428,000 inhabitants in 2010 [17,18]. Butantã is an area with marked socioeconomic inequalities; although its mean family income is higher than the city's average, 13.1% of its inhabitants live in favelas or shanty towns (São Paulo city average, 11.1%) [18]. The HU-USP hospital assists patients with ACS in a frequency of one patient per day. They are treated in the emergency department, internal medicine ward, and/or in the intensive care unit during the hospitalization. However, this is a non-specialized community hospital, therefore, it don't perform procedures like percutaneous coronary intervention (PCI) or revascularization. These procedures are practiced in a cardiology referral center, Instituto do Coração (InCor), eight kilometers away from the community hospital, characterized by uninterrupted emergency services and specialized cardiology care.

2.2. Acute coronary syndrome definition

Medical staff identified possible patients with suspected ACS at the emergency department of HU-USP. Cardiac troponin I (cTnI) was measured in all patients at hospital admission by the index event. Whenever an ACS event is suspected, the clinical protocol in our hospital is to determine troponin levels at entrance and repeat them when normal after usually 6–9 h.

Two different kits were applied to measure cTnI in the study: 1)Abbot Axsym (Troponin-I ADV, B8K190), using MEIA (Microparticle Enzyme 146 Immunoassay) and 2)Advia centaur Siemens (Acridinium Ester-based ADVIA Centaur 147 TnI-Ultra Assay) using Chemiluminescence. The analytical range of cTnI was 0.27–4.00 μg/l and 0.01–50.00 μg/l for Axysm and Advia, respectively. Further, the coefficient of variation (CV) was 5.10–7.40% for Axysm and 4.47–7.45% for Advia. In the main analyses, the cut-off points for the myocardial infarction diagnosis were 0.4 μg/l and 0.06 μg/l for Abbot Axsym and Advia centaur Siemens assays, respectively.

All the selected ERICO participants must fulfill diagnostic criteria for ST elevation myocardial infarction (STEMI), non-ST elevation myocardial infarction (NSTEMI) or unstable angina (UA) as described below [16,19].

1) Myocardial infarction: presence of symptoms consistent with cardiac ischemia within 24 h of hospital presentation and levels of cardiac troponin I (cTnI) above the aforementioned cut-offs.

1a) ST elevation myocardial infarction: presence of criteria for MI plus one of the following: persistent ST segment elevation equal to or > 1 mm in two contiguous electrocardiographic leads or the presence of a new or presumably new left bundle branch block.

1b) Non-ST elevation myocardial infarction: presence of criteria for myocardial infarction but not ST elevation myocardial infarction.

2) Unstable angina: symptoms consistent with cardiac ischemia 24 h prior to hospital admission, absence of myocardial infarction criteria and at least one of the following: history of coronary heart disease; positive coronary disease stratification test (invasive or noninvasive); transient ST segment changes equal or > 0.5 mm in two contiguous leads, new T-wave inversion equal to or granter than 1 mm and/or pseudonormalization of previously inverted T-waves or diagnostic concordance of two independent doctors.

2.3. Study protocol

Participants were interviewed during hospital admission due to ACS and data regarding sociodemographic factors; main cardiovascular risk factors (previous history of hypertension, diabetes, obesity, dyslipidemia, smoking, familial and personal history of coronary heart disease, physical inactivity, cocaine use and menopause) and medications were collected according to standardized questionnaire [16]. Three physicians were responsible for reviewing patient information and for validating ACS cases. By study protocol, a blood sample was drawn for laboratory tests such as N-TYR, and lipid profile that included total cholesterol (Total-Chol), HDL and LDL-cholesterol (HDL-C and LDL-C) and triglycerides (TG). All the laboratory analyses were performed at a single laboratory. Lipids were taken at acute phase (within 24 h of hospital admission to 10 days after ACS) and subacute phase (30 days after ACS). During hospital admission for ACS, participants were undergoing to coronary angiography and then diagnosed as having coronary obstruction if > 50% of obstruction was detected.

At 30 days after the event, participants were invited for a new in-site evaluation by a physician for updating information on cardiovascular risk. This evaluation included clinical data, blood sample and echocardiogram. At six months and annually after the index event, all participants are contacted by phone to update information about their vital status, cardiovascular history and medications.

2.4. Lipids measurements

Lipids analyses were carried out using an automated analyzer. For measurement of T-Chol, HDL-cholesterol, and triglycerides, enzymatic colorimetric assay (ADVIA 1200, Siemens) was used; LDL-cholesterol was calculated using Friedewald equation except for cases with elevated triglyceride levels when a enzymatic colorimetric assay was used (ADVIA 1200, Siemens). The analytical ranges for total-Chol, LDL and HDL-C and TG were 0–17.48 mmol/l, 0–25.90 mmol/l, 0–2.98 mmol/l and 0–6.21 mmol/l, respectively.

For both hs-cTnI and lipids, blood samples were collected in non-anticoagulation gel separator vacuum tubes with 5 ml to obtain serum. The blood samples collected at hospital admission due to ACS were centrifuged for 15 min at 5° degrees Celsius at a speed of 3500 rpm. The serum collected after 30 days ACS was frozen at −80 °C for posteriori analysis.

2.5. Nitrotyrosine measurements

For nitrotyrosine analyses, blood samples were collected in non-anticoagulation gel separator vacuum tubes with 5 ml to obtain serum. The serum was aliquoted and frozen at −80 °C with a single freeze-thaw cycle before biomarker analysis. The volume of the serum was a total of 200 μl (100 μl per well for each patient because we did analyze in duplicate). Samples were collected at admission from 24 h to 10 days from onset of ACS symptoms. N-TYR concentration was analyzed using colorimetric Enzyme-Linked Immunosorbent Assay (ELISA), following the kit instructions (HK501; Hycult Biotech Inc., Plymouth Meeting, PA, USA). Analytical performances of ELISA test have been analyzed during the study. Nitrotyrosine concentration was determined by interpolation on a standard curve. In this kit, the wavelength used was 450 nm, measured with a spectrophotometer. The analytical range of N-TYR is from 2 nmol/l to 1500 nmol/l. By the manufacturer's assay, imprecision of the nitrotyrosine was < 15% of the mean value. Further, we evaluated our samples and it was found a lower variation (mean

variation < 8%). Of note, in the ERICO study we found a small proportion of samples that were out (> 1500 nmol/l) of this standard range of N-TYR concentrations (8 cases, 1.86%). These cases were excluded from the present analysis; thus, they were neither diluted nor retested for an exact quantification. Finally, there is no reference range defined for N-TYR according to previous literature [8,11,15,20,21].

2.6. Outcomes

Here, we analyzed three fatal endpoints: all-cause, cardiovascular and CHD mortality. Vital status was investigated periodically by a hot-pursuit strategy during the follow-up. Mortality information (death certificates) was yearly confirmed by the following official sources: the municipal ("Programa de Aprimoramento das Informações de Mortalidade no Município de São Paulo, PRO-AIM"), the State ("Fundação Sistema Estadual de Análise de Dados-SEADE") and the Brazilian Ministry of Health offices. On a regular basis, the research team prepared a list of all individuals who had died or with whom contact had been lost. The official sources performed a search of their files to obtain death certificates and returned the results of this search to the ERICO research team. Two medical doctors reviewed these data and classified the cause of death for deceased participants according to the information from death certificates. If necessary, a third doctor analyzed the death certificate followed by a consensus meeting. Participants were defined to have died from a cardiovascular cause (cardiovascular mortality) if we identified a cause of death classified in the 10th version of the International Classification of Diseases (ICD-10) chapter IX "Diseases of the circulatory system" or if we identified a cause of death classified with the ICD-10 code R57.0 "Cardiogenic shock". Each identified event was adjudicated using predefined international criteria [22,23].

The study protocol was approved by the Institutional Review Board addressing research in human participants. All participants provided written informed consent for the study. Further details about ERICO study are elsewhere [16].

2.7. Statistical analysis

Descriptive analyses of ERICO participants, including lipids, were demonstrated according to N-TYR tertiles. Categorical variables, which were presented in absolute values and frequencies, were analyzed by Chi-square test. We performed the normality test of Kolmogorov-Smirnov to verify the distribution of continuous variables, which present no parametric distribution and, thus, they were presented in median with respective interquartile ranges (IQR) and analyzed by Kruskal-Wallis test. Of note, the variance of continuous variables across subgroups (N-TYR tertiles) was similar.

Case-fatality rates were evaluated according to tertiles of N-TYR levels at 180 days, one, two and four years after ACS. All-cause, cardiovascular and CHD mortality were evaluated by Kaplan Meyer survival curves and Cox regression logistic regression models were used to calculate the cumulative hazard ratios (HR) with respective 95% confidence interval (95%CI) from 180, 1, 2 and 4 years of follow-up. We calculated logistic presented as: crude (model 1), adjusted for age-sex (model 2), adjusted for age, sex, LDL-cholesterol and HDL-cholesterol levels (model 3). Additional adjustment for other confounders such as ACS subtypes, body mass index, smoking, diabetes, and hypertension and coronary obstruction (none, one, two or ≥ 3 arteries) were also tested.

All the tests were two-tailed with a significance of < 0.05. All statistical analyses were performed by statistical program SPSS® Statistics version 24.0 available from IBM®.

Based on a previous study that evaluated CHD survival associated with nitrotyrosine levels [15], it was calculated that a minimum sample of 288 participants would be needed (including 20% for loss to follow-up) at a statistical power of ≥0.80 and a value of $p < .05$.

Table 1
Baseline characteristics of the 342 participants from ERICO cohort, according to nitrotyrosine tertile.

Characteristics	Nitrotyrosine tertile			P-value
Sociodemographic	1st (n = 113)	2nd (n = 111)	3rd (n = 114)	
Median age, years (IQR)	62 (54–70)	59 (51–70)	61 (53–69)	0.35
Men (%)	69 (60.5)	77 (67.5)	57 (50.0)	0.03
Race (%)				0.11
White	74 (64.9)	74 (66.1)	69 (60.5)	
Brown	36 (31.6)	29 (25.9)	38 (33.3)	
Black	4 (3.5)	5 (4.5)	7 (6.1)	
Asian	–	4 (3.6)	–	
Marital status (%)				0.79
Single	20 (17.5)	15 (13.3)	22 (19.5)	
Married	69 (60.5)	74 (65.5)	65 (57.5)	
Divorced	9 (7.9)	8 (7.1)	6 (5.3)	
Widowed	16 (14.0)	16 (14.2)	20 (17.7)	
Education (%)				0.22
No formal education	14 (12.3)	18 (15.8)	14 (12.3)	
Elementary	71 (62.3)	57 (50.0)	77 (67.5)	
High-school	19 (16.7)	27 (23.7)	15 (13.2)	
College	10 (8.8)	12 (10.5)	8 (7.0)	
Cardiovascular risk factors				
Smoking (%)				0.13
Current	30 (27.0)	39 (35.1)	32 (28.3)	
Past	38 (34.2)	33 (29.7)	50 (44.2)	
Never	43 (38.7)	39 (35.1)	31 (27.4)	
Mean body mass index, kg/m^2 (IQR)	27 (24–30)	26 (23–29)	27 (24–30)	0.39
Obesity (%)	31 (27.7)	25 (22.1)	27 (23.9)	0.61
Hypertension (%)	88 (79.3)	86 (77.5)	85 (75.2)	0.77
Diabetes (%)	46 (43.0)	31 (29.0)	39 (34.8)	0.10
Dyslipidemia (%)	58 (56.3)	49 (49.0)	53 (50.5)	0.54
Sedentarism (%)	83 (74.8)	70 (64.2)	79 (71.2)	0.22
Previous coronary heart disease (%)	31 (29.2)	21 (19.4)	27 (25.2)	0.25
Previous medication (%)				
Aspirin	40 (38.1)	31 (29.8)	33 (29.7)	0.33
Lipid-lowering drugs	26 (24.8)	23 (22.1)	26 (23.4)	0.90
Fibrate	–	4 (3.8)	2 (1.8)	0.12
Antiinflammatory drugs	2 (1.9)	1 (1.0)	1 (0.9)	0.77
Corticosteroid	6 (5.7)	7 (6.7)	7 (6.4)	0.95
Immunosuppressive drugs	–	1 (1.0)	2 (1.8)	0.39
Types ACS (%)				
Angina	21 (18.4)	18 (15.8)	15 (13.2)	0.13
NSTEMI	68 (59.6)	55 (48.2)	69 (60.5)	
STEMI	25 (21.9)	41 (36.0)	30 (26.3)	
Coronary obstruction (%)				
None	14 (15.7)	15 (16.3)	16 (16.7)	0.91
1 artery	38 (42.7)	42 (45.7)	39 (40.6)	
2 arteries	17(19.1)	19 (20.7)	24 (25.0)	
≥3 arteries	20 (22.5)	16 (17.3)	17 (17.7)	

P-values were obtained from Chi-square test for categorical variables and from Kruskal-Wallis for continuous variables.
IQR: interquartile range.
NSTEMI: non-ST elevation myocardial infarction; STEMI: ST elevation myocardial infarction.
Coronary artery obstruction was considered when > 50% of obstruction was detected by angiography.

3. Results

3.1. General findings

Among 342 participants, the median value of N-TYR levels was 208.33 nmol/l (range: 3.09 to 1500.00 nmol/l). For the 1st tertile the median value for N-TYR levels was 51.73 nmol/l (range: 3.09 to 124.91 nmol/l), for the 2nd tertile was 208.33 nmol/l (range: 125.36 to 346.67 nmol/l) and for 3rd tertile was 581.75 nmol/l (range: 346.73 to 1500.00 nmol/l). Although N-TYR levels collected within 24 h after acute event were lower than those collected > 24 h, we did not find

statistical difference regarding N-TYR levels (164.90 vs. 218.35 nmol/l, p = 0.36).

Baseline characteristics of sample according to N-TYR tertiles are shown in Table 1. In this sample (median age: 60 y-old) almost 60% were male, most of the participants were white (63.8%), married (61.2%) and had at most elementary education (59.9%). Regarding sociodemographic characteristics, we found a significant difference in the proportion of men across N-TYR tertiles (p = 0.03). Other characteristics, as well as, frequencies of cardiovascular risk factors (CVRF), use of previous medication and ACS subtype did not differ across N-TYR tertiles.

Since we used two different Tn assays (Abbot Axsym® and Advia centaur Siemens®) in the present study, we performed a sensitivity analysis that attested no significant difference in the ACS cases distribution according to Tn assays used in the present study (p = 0.21).

3.2. Lipids findings

Overall, we noticed progressively lower levels of T-Chol, LDL-C and triglycerides in the acute compared to post-acute phase after ACS. HDL-C levels remained almost the same across periods after ACS (Table S1). We also performed a comparative analysis of lipids profile (T-Chol, LDL-C, HDL-C, TG and CT: HDL ratio), taken at acute (up to10 days after ACS) and subacute phase (30 days after ACS), across N-TYR tertiles to better evaluate the link between this inflammatory marker, lipids and the causality of future cardiovascular events (Table 2). At acute phase, we noticed higher levels of T-Chol in the 2nd N-TYR tertile, p = 0.03. Also, LDL cholesterol was significant when dosed 2–10 days from hospital admission of ACS at acute phase, in the 2nd N-TYR tertile, p = 0.04. Other lipids were not associated with N-TYR distribution.

3.3. Case-fatality

Table 3 shows case-fatality evaluated according to tertiles of N-tyr levels at 180 days, one, two and four years after ACS. We found no statistical differences for case-fatality rates and N-TYR distribution.

3.4. Mortality and survival across nitrotyrosine levels

During follow-up of 4 years, we observed 44 (12.9%) deaths (5.6% due to CVD and 1.8% was due to CHD). Survival rate was 87.1% (Survival days 1352, 95%CI: 1320–1387 days). We found no statistical differences in all-cause, CVD and CHD mortality according to tertiles of N-TYR levels (Fig. 1).

Cox regression analyses confirmed our findings regarding the lack of association of N-TYR levels and mortality (all-cause, CVD and CHD) from 180 days to 4 years of follow-up (Table 4). The additional adjustment for other confounders such as ACS subtypes, body mass index, smoking, diabetes, hypertension and coronary obstruction did not modify our findings.

4. Discussion

Among these participants from ERICO cohort, N-TYR levels did have any association on the mortality risk (all-cause, CVD and CHD) four years of follow-up after ACS, despite the pathophysiologic mechanism of oxidative atherosclerosis process in which N-TYR is supposedly involved.

Recently, N-TYR has been implicated in atherosclerosis process, by inflammatory pathway playing a primordial role in the oxidation of lipids, oxidative damage/cell injury and in the oxidative modification of low-density protein (LDL) into a proatherogenic form causing an endothelial dysfunction of coronary arteries [8–11,15,24–26]. Oxidative stress ended up in an excessive production of reactive oxygen and nitrogen species such as peroxynitrite [26–28]. Peroxynitrite, which is abundant in atherosclerotic lesions and can be quantified by N-TYR serum levels [29], activates or accelerates atherogenesis by the atherosclerotic plaque rupture causing the myocardial infarction [30,31]. Therefore, N-TYR, a stable end product of nitration of tyrosine by peroxynitrite, which is generated by nitric oxide (NO) in combination with reactive oxygen species, has been suggested to be useful as a marker for NO-mediated tissue damage [27,28,32].

Some previous studies have reported the relationship between N-TYR and CHD but with no consensus [8,11,33]. Tanabe et al. [11] performed a case-control study with 30 Japanese patients (14 cases with suspected vasospastic angina pectoris (VSAP): 16 controls) to

Table 2
Lipids distribution during acute and late phase after acute coronary syndrome among 342 participants from ERICO cohort according to nitrotyrosine tertile.

Characteristics	Nitrotyrosine tertile			P-value
	1st	2nd	3rd	
Lipids dosed within 24 h from hospital admission of ACS				
Total cholesterol, mmol/l	5.00 (4.27–5.92)	4.89 (4.11–5.66)	4.58 (3.67–5.33)	0.16
LDL cholesterol, mmol/l	3.08 (2.51–3.85)	3.03 (2.28–3.75)	2.66 (2.04–3.54)	0.16
HDL cholesterol, mmol/l	1.06 (0.91–1.24)	0.96 (0.83–1.22)	0.96 (0.80–1.16)	0.62
TC:HDL cholesterol ratio	4.57 (3.93–6.19)	4.89 (3.74–5.88)	4.62 (3.76–5.63)	0.74
Triglycerides, mmol/l	1.63 (1.16–2.07)	1.50 (1.06–2.35)	1.30 (0.97–2.22)	0.63
Lipids dosed 2–10 days after hospital admission of ACS				
Total cholesterol, mmol/l	4.27 (3.41–5.04)	4.89 (3.65–5.72)	4.24 (3.47–5.09)	0.03
LDL cholesterol, mmol/l	2.53 (1.78–3.13)	2.97 (2.20–3.65)	2.53 (2.04–3.23)	0.04
HDL cholesterol, mmol/l	0.93 (0.78–1.11)	0.93 (0.75–1.16)	0.91 (0.72–1.19)	1.00
TC:HDL cholesterol ratio	4.50 (3.42–5.34)	5.13 (4.05–6.77)	4.63 (3.64–5.79)	0.12
Triglycerides, mmol/l	1.43 (1.13–1.93)	1.70 (1.20–2.30)	1.37 (1.13–1.96)	0.30
Lipids dosed 30-days after hospital admission of ACS				
Total cholesterol, mmol/l	3.78 (3.16–4.56)	3.73 (3.32–4.25)	3.76 (3.16–4.45)	0.88
LDL cholesterol, mmol/l	2.10 (1.55–2.49)	2.10 (1.61–2.51)	1.99 (1.53–2.62)	0.99
HDL cholesterol, mmol/l	1.01 (0.62–1.22)	1.01 (0.85–1.17)	0.93 (0.83–1.22)	0.60
TC: HDL cholesterol ratio	3.72 (3.10–4.50)	3.79 (3.07–4.32)	3.70 (3.17–4.49)	0.99
Triglycerides, mmol/l	1.41 (1.04–1.89)	1.33 (1.01–1.84)	1.34 (1.04–1.74)	0.77

Some proportions might not add up to 100% due rounding or missing values (at most 8.8% in LDL and HDL cholesterol 10 days after acute phase).
Lipids are presented in median and interquartile ranges (IQR).
P-values were obtained from Kruskal-Wallis.
ACS: Acute coronary syndrome.
LDL cholesterol: low-density lipoprotein cholesterol; HDL cholesterol: high-density lipoprotein protein; TC: HDL cholesterol ratio.

Table 3
Case-fatality at 180-days, 1-year, 2-year and 4-year in the ERICO cohort, according to nitrotyrosine levels tertiles.

	180-days (deaths/total)	1-year (deaths/total)	2-year (deaths/total)	4-year (deaths/total)
	(7/340)	(12/339)	(25/329)	(44/264)
Nitrotyrosine tertiles (range)				
1st	4/113 (3.5)	6/112 (5.4)	13/108 (12.0)	16/85 (18.8)
2nd	2/113 (1.8)	3/113 (2.7)	5/110 (4.5)	12/94 (12.8)
3rd	1/114 (0.9)	3/114 (2.6)	7/111 (6.3)	16/85 (18.8)

P-values were obtained from Chi-square test.
*P-value < 0.001 comparing dead and alive participants.
**P-value < 0.05 comparing dead and alive participants.

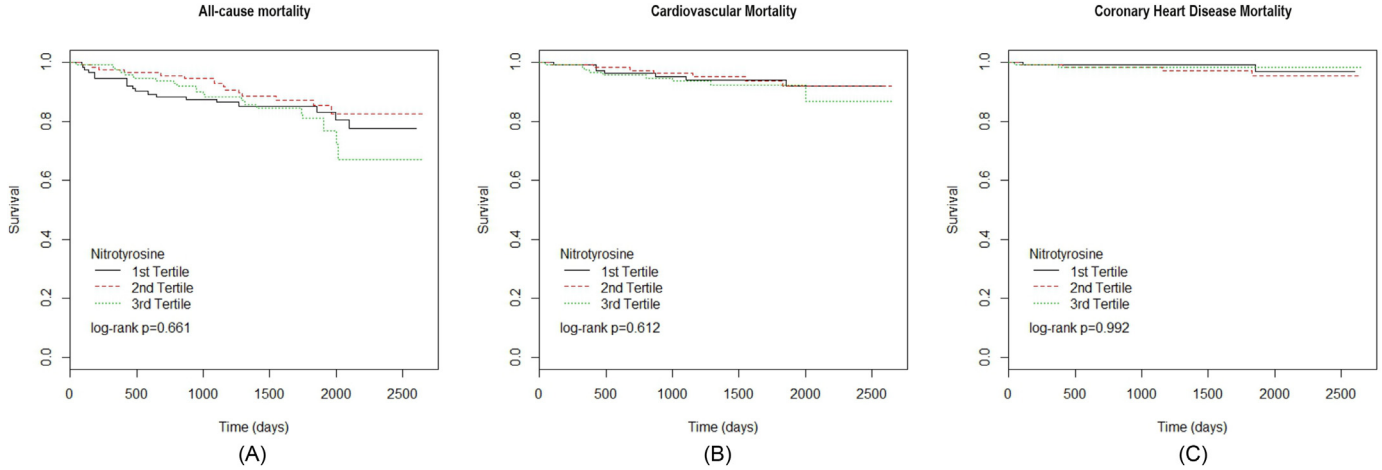

Fig. 1. Nitrotyrosine levels as predictor all-cause survival (A), cardiovascular (B) and coronary heart disease (C) in participants from ERICO study during a follow-up of 4 years.

evaluate the complex association between myocardial ischemia and N-TYR levels. It was observed a statistically significant difference on N-TYR levels at 3 to 12 h after coronary vasospasm induced by intracoronary acetylcholine test provocation in the subgroup of cases (11.3 ± 3.3 μg/ml at 3 h, p = 0.015,12.1 ± 5.7 μg/ml at 12 h, p = 0.03). On the other hand, levels of N-TYR decreased significantly in the VSAP-negative group after provocation test [11]. Shishehbor et al. [8] investigated N-TYR levels in a case-control in 208 patients with chronic CAD (100 cases: 108 controls). All cases were classified as having established CAD, defined as myocardial infarction, coronary artery bypass graft surgery, percutaneous coronary intervention, or a stenosis of 50% or greater in 1 or more major coronary vessels on angiography. It was showed significant increased levels of nitrotyrosine among CAD patients (n = 100, median 9.1 μmol/mol p < 0.001) compared with controls (n = 108, median 5.2 μmol/mol p < 0.001). In contrast, one case-control study that evaluated the relationship between N-TYR and CHD reported similar null findings [33]. In this study, 63 individuals (29 cases: 34 controls) were undergoing an ETT (exercise tolerance test) with induced myocardial ischemia, and had N-TYR levels collected 15 min before and 30 min after the ETT. N-TYR levels were similar in patients with electrocardiographic myocardial ischemia and in controls before and after exercise, suggesting that N-TYR does not contribute to myocardial ischemia induced by exercise [33].

In our study, N-TYR collected up to 10 days did not differ across ACS subtype. Although the N-TYR levels among those who had their blood collected with < 24 h were higher compared to those collected with > 24 h, this difference was not statistically significant.

There is only one study involving N-TYR levels and ACS mortality [15]. Heslop et al. [15] evaluated a prospective cohort of coronary angiography patients (n = 885), with 651 having an angiographic CAD (604 with severe lesions: ≥50% stenosis) who had their N-TYR levels dosed at this time. All participants classified as having stable angina, myocardial infarction (MI) or even other cardiovascular diseases (such as aortic stenosis and\or regurgitation) were followed-up during 13 years. Of note, those with unstable angina or MI within 2 months preceding the study were excluded [15]. It was observed that risk of cardiovascular mortality increased across N-TYR tertiles (p = 0.029) with significant differences between highest and lowest tertiles appearing after 4 years of follow-up. It was reported an increased cardiovascular mortality risk for those in the 2nd tertile of N-TYR (crude HR, 1.87; 95% CI: 1.15–3.13) compared with the 1st tertile, but not for the 3rd tertile. However, after the multivariate adjustment for age, sex, total/high-density lipoprotein cholesterol ratio, body mass index, smoking, diabetes, and hypertension, in this long-term mortality risk was attenuated (p = 0.08) [15].

Similar to Heslop et al. study [15], we did not observe N-TYR levels as independent predictor of mortality after ACS four years of follow up. We could explain the lack of association of N-TYR with our fatal outcomes after ACS by the relatively low levels of T-Chol and LDL-C in the acute to late phase after ACS, added to the use of lipid-lowering medications. Further, any significant association of inflammation through lipids and N-TYR tertiles were established. Although there is evidence that lipid-lowering drugs down regulated N-TYR systemic levels in patients with acute coronary syndrome [8], we found no significant association with lipid-lowering drugs and N-TYR tertiles. Nevertheless, we cannot refute the possibility of inflammation/oxidation process interfering in N-TYR levels and consequently in its relation with CVD mortality [15]. Indeed, blood samples for N-TYR were collected at hospital admission within 24 h to 10 days after the onset of symptoms. It has been described the increased in N-TYR levels at 4 h and peaked at 24 h after percutaneous coronary intervention [34]. However, in our study N-TYR levels did not differ among those who had their levels

Table 4
Hazard ratios (HR) [95% Confidence Intervals (95% CI)] of all- cause, CVD mortality, and CHD mortality in the participants from ERICO cohort according to nitrotyrosine distribution.

Variation of nitrotyrosine levels tertiles Hazard ratios (95%CI)	180 days	1 year	2 years	4 years
All-cause mortality				
Crude				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	0.50 (0.09–2.71)	0.49 (0.12–1.97)	0.37 (0.13–1.04)	0.69 (0.33–1.46)
3rd	0.25 (0.03–2.21)	0.49 (0.12–1.94)	0.52 (0.21–1.29)	0.96 (0.48–1.91)
Age and sex-adjusted				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	0.54 (0.10–2.93)	0.53 (0.13–2.12)	0.40 (0.14–1.13)	0.75 (0.36–1.59)
3rd	0.26 (0.03–2.36)	0.51 (0.13–2.07)	0.53 (0.21–1.33)	0.96 (0.48–1.94)
Multivariable adjusted				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	1.00 (0.15–6.99)	0.70 (0.16–2.98)	0.47 (0.16–1.35)	0.78 (0.35–1.71)
3rd	0.39 (0.04–3.96)	0.57 (0.13–2.41)	0.58 (0.23–1.51)	0.95 (0.46–1.98)
CVD mortality				
Crude				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	1.00 (0.06–15.94)	0.99 (0.06–15.79)	0.72 (0.16–3.21)	0.78 (0.24–2.55)
3rd	0.99 (0.06–15.85)	2.93 (0.30–28.12)	1.20 (0.32–4.47)	1.28 (0.44–3.68)
Age and sex-adjusted				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	1.08 (0.07–17.32)	1.07 (0.07–17.12)	0.78 (0.18–3.51)	0.85 (0.26–2.79)
3rd	0.91 (0.06–14.82)	2.81 (0.29–27.32)	1.22 (0.33–4.59)	1.35 (0.46–3.91)
Multivariable adjusted				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	1.96 (0.09–44.29)	1.14 (0.07–18.82)	0.76 (0.17–3.40)	0.82 (0.25–2.69)
3rd	1.30 (0.07–23.38)	2.84 (0.29–27.88)	1.13 (0.30–4.24)	1.08 (0.36–3.23)
CHD mortality				
Crude				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	1.00 (0.06–15.94)	1.00 (0.06–15.94)	1.95 (0.18–21.53)	2.87 (0.30–27.56)
3rd	0.99 (0.06–15.85)	0.99 (0.06–15.85)	1.95 (0.18–21.54)	1.96 (0.18–21.62)
Age and sex-adjusted				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	1.08 (0.07–17.32)	1.07 (0.07–17.32)	2.12 (0.19–23.37)	3.20 (0.33–30.73)
3rd	0.91 (0.06–14.82)	0.91 (0.06–14.82)	1.92 (0.17–21.62)	2.01 (0.18–22.46)
Multivariable adjusted				
1st	Reference (1.0)	Reference (1.0)	Reference (1.0)	Reference (1.0)
2nd	1.96 (0.09–44.29)	1.96 (0.09–44.29)	2.70 (0.22–33.80)	4.57 (0.40–52.20)
3rd	1.30 (0.07–23.38)	1.30 (0.07–23.38)	2.16 (0.18–25.32)	2.41 (0.21–28.19)

Multivariate adjusted by age, sex, LDL-cholesterol and HDL-cholesterol levels.
HR: hazard ratio.
CVD: cardiovascular; CHD: coronary heart disease.

collected with < 24 h compared to those with > 24 h. Finally, the lack of association between mortality and N-TYR levels after ACS in our study could be justified by the severity of CAD, since more severe cases are transferred to a cardiology referral center (InCor), in the first hours of onset ACS symptoms after hospital admission. Of note, even analyzing N-TYR and mortality in different times and follow-up than the study of Heslop et al. [15] that evaluated more stable CAD (13 years), we obtained similar findings, which reinforces the participation of N-TYR in those individuals with more severe coronary lesions who had more oxidative stress and consequently more damage in myocardial.

Our previous findings extend and refine the current evidence implicated in the oxidative stress mediated by N-TYR in cardiovascular mortality; however, we still have a lacking of knowledge in this complex relationship.

Our study has some strength. This a unique study that evaluated prognostic value of N-TYR levels as independent predictors of short- and long-term mortality, including all-cause, CVD and CHD mortality performed in a prospective cohort of ACS in a middle income country.

The main limitation of our study is the fact that we were not able to quantify N-TYR in the entire cohort, which could have introduced a bias into this prospective study. Although previous publication in the same cohort demonstrated similar baseline characteristics compared to the current sample analyzed here [16,35]. Also, in this setting, more severe cases of ACS, which could have more altered levels of N-TYR, were transferred to our cardiology referral center, Instituto do Coração (InCor). Thus, this fact could have had some influence our null findings regarding mortality and N-TYR levels.

Another possible reason for the lack of association between nitrotyrosine levels and ACS mortality could has been an underpowered sample to detect this effect. However, we did calculate a minimum sample power of 288 to detect this effect and, in fact, we analyzed a bigger sample than that ($N = 342$). Finally, the various methods such as chromatography and ELISA used to measure nitrotyrosine have some important pitfalls that have difficulted the monitoring of nitrotyrosine in biological fluids and consequently comparisons across the studies. Therefore, the development of a method that is reliable, sensitive, selective and appropriate to N-TYR analysis is needed [27,36,37].

5. Conclusion

In the ERICO study, N-TYR levels did not demonstrate to be an independent predictor of short- and long-term mortality after ACS during 4-years of follow-up. Future research should be guaranteed to elucidate nitrotyrosine as potential prognostic marker of cardiovascular mortality

after acute coronary event.

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