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# Association of proprotein convertase subtilisin/kexin type 9 (PCSK9) levels with abnormally high ankle-brachial index in atrial fibrillation

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**Association of proprotein convertase subtilisin/kexin type 9 (PCSK9) levels with abnormally high ankle-brachial index in atrial fibrillation**

**Short title:** PCSK9, ABI and mortality risk

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**WHAT'S NEW?**

Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) is associated with abnormally high ankle-brachial index (ABI) ≥1.4 in anticoagulated atrial fibrillation patients. High serum PCSK9 and ABI ≥1.4 are associated with increased mortality risk in this patients' population.

**ABSTRACT**

**Background:** High ankle-brachial index (ABI) has been associated with increased risk of

worse outcomes in the general population. Few data on atrial fibrillation (AF) do exist. Experimental data suggest that proprotein convertase subtilisin/kexin type 9 (PCSK9) contribute to vascular calcification but clinical data on this association are lacking.

**Aims:** We want to investigate the relationship between circulating PCSK9 levels and abnormally high ABI in patients suffering from AF.

**Methods:** We analysed data from 579 patients included in the prospective ATHERO-AF study. An ABI ≥1.4 was considered as high. PCSK9 levels were measured coincidentally with ABI measurement. We used an optimized cut-offs of PCSK9 for both ABI and mortality obtained from ROC curve analysis. All-cause mortality according to the ABI value was also analysed.

**Results:** 115 (19.9%) had an ABI ≥1.4. The mean (SD) age was 72.1 (7.6) years and 42.1% of patients were women. Patients with ABI ≥1.4 were older, more frequently male and diabetic. Multivariable logistic regression analysis showed an association between ABI ≥1.4 and serum levels of PCSK9 >1150 pg/ml (odds ratio [OR], 1.649; 95% confidence interval [CI], 1.047–2.598; *P* = 0.031). During a median follow up of 41 months, 113 deaths occurred. At multivariable Cox regression analysis, ABI ≥1.4 (hazard ratio [HR], 1.626; 95% CI, 1.024–2.582; *P* = 0.039), $CHA_2DS_2$-VASc score (HR, 1.249; 95% CI, 1.088–1.434; *P* = 0.002), antiplatelet drug use (HR, 1.775; 95% CI, 1.153–2.733; *P* = 0.009), and PCSK9 >2060 pg/ml (HR, 2.200; 95% CI, 1.437–3.369; *P* <0.001) were associated with all-cause death.

**Conclusions:** In AF patients, PCSK9 levels relate to an abnormally high ABI ≥1.4. Our data suggest a role for PCSK9 in favouring vascular calcification in AF patients.

**Key words:** ABI, PCSK9, mortality, atrial fibrillation, peripheral artery disease

## INTRODUCTION

Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9) is an enzyme involved in cholesterol metabolism inducing intracellular degradation of hepatic low-density lipoprotein (LDL) receptors, reducing serum LDL[1].

In addition to its cholesterol-related actions, PCSK9 may represent a risk factor for cardiovascular disease through several mechanisms, including reactive oxygen species generation [2], platelet activation [3] and in direct modifications of blood vessels walls [4], contributing to an accelerated vascular calcification [4–6].

PCSK9 has been found to be increased in some cardio-metabolic diseases such as

hypertension and diabetes mellitus and to be associated with an increased risk of cardiovascular events (CVEs) in some cardiovascular settings, including atrial fibrillation (AF) [3].

In particular, AF patients are characterized by an atherosclerotic burden, with a high prevalence and incidence of coronary artery disease and peripheral arterial disease (PAD) [7]. The prevalence of PAD is difficult to estimate, given a high proportion of undiagnosed patients. Currently, it is estimated that a population of >200 million patients worldwide suffer from PAD [8]. PAD carries an increased risk of CVEs, major adverse limb events including vascular re-intervention and deaths [9–11], leading a reduction of quality of life and life expectation. For this, reason, European Society of Cardiology (ESC) Guidelines [12] recommend to screen patients at risk for PAD by the ankle brachial index (ABI), which represents an evidence-based, useful, and low-cost screening tool for asymptomatic PAD. In addition to its diagnostic value, ABI has also prognostic implications, as either low (≤0.9) or high (>1.3) ABI has been associated with an increased risk of CVEs and cardiovascular death [13].

Indeed, also in the AF population, a previous prospective study showed an increased risk of CVEs in patients with impaired ABI <0.9 compared to normal ABI (0.9–1.3) [14].

However, few data on clinical characteristics of AF patients with high ABI >1.3 have been reported so far.

Based on this, we investigated the relationship between circulating PCSK9 levels and abnormally high ABI in patients suffering from AF enrolled in the prospective ATHERO-AF study.

## METHODS

We analysed data on patients enrolled in the prospective, observational ATHERO-AF cohort of consecutive patients with non-valvular AF at the Department of Clinical, Internal, Anaesthesiologic and Cardiovascular Sciences, Sapienza University of Rome. The ATHERO-AF study started in February 2008 and is still ongoing (ClinicalTrials.gov Identifier: NCT01882114). Since 2013, the study protocol has been amended to include a second branch of patients treated with direct oral anticoagulants. The current analysis is performed on the branch of patients on vitamin K antagonists (VKAs). Patients are regularly monitored every 2-3 weeks according to INR values. The follow-up of each patient is stopped when one of the primary endpoints occurs (as previously defined in [15]).

Quality of anticoagulation was calculated by the time in therapeutic range (TiTR) as

previously described [16]. During the first clinical examination a complete personal medical history was collected, including drug therapy and comorbidities.

**Cardiovascular risk factors**

Definitions of cardiovascular risk factors such as hypertension, diabetes mellitus and heart failure have been previously reported and defined according to international guidelines [17–20].

Optimized risk factors were defined according to the ABC pathway, as previously described [21]. Thus, well-controlled hypertension is defined as blood pressure <140/90 mm Hg, a standard of care for the management of heart failure is considered the treatment with angiotensin converting enzyme inhibitors (ACE-I) or angiotensin receptor blockers (ARB) [21]. Optimized treatment for previous myocardial infarction/coronary revascularization was considered the use beta-blockers[21].

**Inclusion criteria**

All patients presenting with non-valvular AF who were >18 years of age were eligible for the study. All patients were treated with VKAs after appropriate thrombotic risk stratification [22] (international normalized ratio target: 2.5).

**Exclusion criteria**

Exclusion criteria were prosthetic heart valves, severe cognitive impairment, chronic infections (human immunodeficiency virus infection, hepatitis C virus, hepatitis B virus), or systemic autoimmune disease. Patients with an ABI <0.9 or overt PAD were excluded.

**Lipid profile and PCSK9 serum levels assessment**

Patients were also asked to collect a blood sample. At baseline, a lipid profile was obtained, including total cholesterol, high-density lipoprotein (HDL) (mg/dl), and triglycerides (mg/dl). Low-density lipoprotein (LDL, mg/dl) cholesterol was calculated by the validated Friedewald formula. Also, creatinine (mg/dl) with glomerular filtration rate (GFR) (ml/min/1.73 $m^2$) by simplified MDRD formula, and fasting blood glucose (mg/dl) were obtained.

Blood samples obtained were taken into tubes with 3.8% sodium citrate and centrifuged at 300 g for 10 min to obtain supernatant, then immediately stored at -80°C until use. Plasma levels of PCSK9 were measured by a commercial enzyme-linked immunoadsorbent assay (Boster Biological Technology 3942 B Valley Ave, Pleasanton, CA 94566) [3]. Plasma

samples were diluted 1:10 in diluent buffer. Data are expressed as pg/ml, and the minimal detectable dose of PCSK9 was <10 pg/ml in human plasma. The assay and interassay coefficients of variance were 5.8% and 6.9%, respectively.

**Ankle-brachial index measurement**

ABI was calculated as the ratio of systolic blood pressure obtained from the ankle and brachial arteries in all AF patients using a 8 MHz CW Vascular Doppler (Risingmed Model:RFD-B). All procedures followed guidelines recommendations[23]. Ankle and brachial systolic blood pressures were measured both in the right and left side, and ABI was assessed separately for the right and left leg using the highest arm and ankle pressures. A value of ABI≥ 1.4 was considered as abnormally high. Patients with ABI <0.9 were excluded, and those with an ABI 0.9-1.3 were used as reference group.

**Statistical analysis**

Categorical variables were reported as number and percentages which were compared by the Pearson chi-squared test. Mean and standard deviation (SD) or median and interquartile range (IQR) were used for continuous variables, which were compared by Student T or Mann-Whitney U test, respectively. Normal distribution of variables was checked by Kolmogorov-Smirnov test. We divided the cohort into two groups according to the ABI values (0.9–1.3 and ≥ 1.4) to compare baseline clinical characteristics. Stepwise logistic regression analysis was used to calculate the odds ratio (OR) and 95% confidence interval (95% CI) test the association between clinical factors and high ABI.

All available variables with complete data were included: sex, age ≥75 years, type of AF, diabetes, smoking, HF, previous stroke/TIA, previous myocardial infarction/coronary revascularization, antiplatelet, ACE inhibitors/sartans, beta blockers, digoxin, proton pump inhibitors (PPI), amiodarone, verapamil, body mass index (BMI). For this model, anti-hypertensive drugs were entered instead of hypertension, and statins instead of cholesterol levels. For the analysis, we used an optimized cut-off of PCSK9 >1150 pg/ml obtained from diagnostic ROC curve analysis (sensitivity 60%, specificity 48%).

Cox proportional hazards regression analysis was used to calculate the adjusted hazard ratio (HR) and 95%CI of all-cause mortality by each clinical variable. For the survival model, we used the composite $CHA_2DS_2VASc$ score instead of single variables given the relative low number of cases. Variables were entered with one step in the multivariable model. Furthermore, we used an optimized cut-off of PCSK9 >2060 pg/ml obtained from ROC curve

analysis for mortality (sensitivity 33%, specificity 84%).

All tests were 2-tailed and only *P*-values <0.05 were considered as statistically significant. The analyses were performed using SPSS 25.0 software (IBM, Armonk, NY, US).

**Sample size calculation**

For this post-hoc analysis we assumed a sampling ratio of 5 to 1 (20% patients with ABI ≥1.4), an HR of 2, and an overall 20% event rate, a sample size of 510 patients guarantees 80% power for a 5% level test. This number was increased to 579 to guarantee a sufficient power and expecting a loss of patients during follow-up of 10%.

**Ethical committee**

All patients signed an informed written consent at study entry. The study was approved by the local ethic committee of Sapienza University (No. 1306/2007) and was conducted according to the Declaration of Helsinki. The study is registered at clinicaltrials.gov NCT01882114.

**RESULTS**

The study enrolled 579 patients with AF, of whom 115 (19.9 %) had an ABI ≥1.4. The mean (SD) age was 72.1 (7.6) years and 42.1% of patients were women (Table 1).

Patients with an ABI ≥1.4 were older than patients with ABI 0.9-1.3 and more frequently men and affected by diabetes. Furthermore, patients with ABI ≥1.4 had a worse control of blood pressure and had a higher thromboembolic risk (Table 1). There was no difference between ABI groups concerning smoking habit, previous cerebrovascular and cardiovascular disease, and concomitant therapies (Table 1).

**PCSK9 and ABI**

In a stepwise multivariable logistic regression analysis, we found an inverse association of female sex and a direct association for diabetes with ABI ≥1.4 (Table 2). We found a significant association between PCSK9 levels >1150 pg/ml and ABI ≥1.4, with an OR 1.649, 95%CI 1.047-2.598, p=0.031).

**ABI, PCSK9 and all-cause mortality**

During a median (IQR) follow up of 41 (23.1–66.1) months, 10 patients in the group of ABI 0.9–1.3 were lost and 113 deaths were registered, 27 in the group of high ABI and 86 in the

group of normal ABI (n = 454). At multivariable Cox regression analysis (Table 3), ABI ≥1.4 was associated with an increased risk of all-cause of death (hazard ratio, 1.626; 95% CI 1.024–2.582; *P* = 0.039). Figure 1 shows the adjusted survival curves according to ABI values from multivariable Cox regression analysis. Other predictors of death were $CHA_2DS_2$-VASc score (HR, 1.249; 95% CI 1.088–1.434; *P* = 0.002), antiplatelet drug use (HR, 1.775; 95% CI, 1.153–2.733; *P* = 0.009), PCSK9 >2060 pg/ml (HR, 2.200; 95% CI, 1.437–3.369; *P* <0.001).

## DISCUSSION

In this prospective study, we found a direct association between PCSK9 levels and abnormally high values of ABI ≥1.4, which was associated with an increased risk of death.

We found that nearly 20% of patients showed an abnormally high ABI ≥1.4. Patients with high ABI were more frequently diabetic, less likely to be women. A well-controlled blood pressure was inversely associated with high ABI. This figure is slightly lower than a previously reported study that included 287 consecutive anticoagulated outpatients with AF, in whom 78 (27%) had an abnormal ABI. In this study, abnormal ABI was associated with diabetes, heart failure and ischaemic heart disease[24]. Similar findings were reported in a prospective study including 5679 subjects, showing that age, female sex and diabetes were associated with high ABI [25].

In addition to this already known factors, we also found that the use of proton pump inhibitors (PPI) was associated with vascular calcification. This finding is in keeping with previous evidence showing that the use of PPI is associated with aortic and iliac arteries vascular calcification [26, 27], increased risk of calcinosis [28]. The mechanism for this association is not completely elucidated but experimental evidence suggested that the use of PPI may influence metalloproteinases (MMPs) activity. In particular, omeprazole induced vascular MMP-2 expression and activity, resulting in an increased media thickness and vascular oxidative stress [29].

A novel finding of our study relies on the significant association between PCSK9 levels and high ABI. A previous experimental study showed that human and rat smooth muscle cells (SMCs), overexpressing PCSK9 showed a higher extracellular calcium deposition compared to control SMCs, when exposed to a pro-calcific environment[30]. Furthermore, the same cells showed an increase in pro-calcific markers, such as bone morphogenetic protein 2 and alkaline phosphatase, and a concomitant decrease in anti-calcific mediator matrix GLA protein and osteopontin [30]. In addition, in asymptomatic patients with familial

hypercholesterolemia, PCSK9 levels were increased in patients with coronary artery calcification as compared to those without [31].

Our findings add to these results providing clinical evidence that PCSK9 may contribute in vivo to vascular calcification also in patients with cardiovascular disease, such as those with non-valvular AF. Patients with AF on treatment with VKAs represent a high-risk group of patients for vascular complications, as VKA therapy itself represent a risk factor for vascular calcification. Hence, previous evidence showed that VKAs were associated with human vascular smooth muscle cells calcification and atherosclerotic plaque progression and increased coronary artery calcification [32–34]. Indeed, the use of VKAs may aggravate vascular calcification in patients with an already abnormally high ABI, especially when elevated PCSK9 levels coexist.

We also analysed the relationship between high ABI and incidence of mortality and found an increased risk of death in AF patients with high ABI. Oure results are in keeping with the study by Velescu et al. [25] in which an ABI ≥1.4 was independently associated with all-cause mortality (HR, 2.0; 95% CI, 1.32–2.92) in subjects without cardiovascular disease at baseline [25]. Also the previously mentioned study by Gallego et al. also showed a similar association with all-cause mortality (adjusted HR, 2·76; 95% CI, 1.08–7.06; $P = 0.033$) in AF patients [24].

Among predictors of mortality, we found that concomitant antiplatelet drug administration is associated with an increased mortality rate. This evidence is in line with previous studies showing that mortality may be the result of increased bleeding events [35–37].

As a clinical implication of the study, further study is needed to investigate if patients with a high ABI may benefit from a switching to a direct oral anticoagulant to reduce the risk of vascular calcification progression, especially if a high PCSK9 concentration is present. In this view, the value of PCSK9 in refining clinical risk in AF patients compared to other biomarkers may be studied [38]. At this regard, the variability of PCSK9 levels over time needs further investigation, as some factors such as high-dose statins may increase PCSK9 levels [39].

Limitations. Our study included elderly Caucasian patients with a relatively low proportion of patients with diabetes. Thus, our results should be confirmed in studies with other ethnicity groups and with a higher percentage of metabolic patients. A second limitation is that patients were anticoagulated with VKAs not allowing to observe if a similar result could be obtained in subjects treated with direct oral anticoagulants. Given the observational design of the study, we can only deduce association but not causality between PCSK9 levels and ABI

values. Furthermore, despite the analysis was adjusted for the most common cardiovascular risk factors, residual confounding is likely to remain. Finally, we used a commercial assay to measure PCSK9 levels, but it cannot discriminate among the various forms of circulating PCSK9.

In conclusion, patients with AF and ABI ≥1.4 have a higher risk of cardiovascular events and disclose high PCSK9 levels. The role of PCSK9 in favouring in vivo vascular calcification needs further investigation.

**Article information**

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**Table 1. Baseline characteristics of patients with atrial fibrillation according to ankle brachial index (ABI) values**

| | **Total cohort (n = 579)** | **ABI 0.9–1.3 (n = 464)** | **ABI ≥1.4 (n = 115)** | ***P*-value** |
|---|---|---|---|---|
| Female sex, n (%) | 244 (42.1) | 213 (45.9) | 31 (27.0) | <0.001 |
| Age, years, mean (SD) | 72.1 (7.6) | 71.6 (7.5) | 74.8 (7.4) | 0.024 |
| Age ≥75 years, n (%) | 233 (40.2) | 184 (39.7) | 49 (42.6) | 0.563 |
| BMI, kg/m$^2$, mean (SD) | 27.5 (4.7) | 27.4 (4.6) | 27.9 (4.9) | 0.339 |
| Persistent/permanent AF, n (%) | 287 (49.6) | 223 (48.1) | 64 (55.7) | 0.145 |
| Well controlled arterial hypertension, n (%) | 134 (23.1) | 118 (25.4) | 16 (13.9) | 0.009 |
| T2DM, n (%) | 104 (18.0) | 67 (14.4) | 37 (32.2) | <0.001 |
| Active smoking, n (%) | 57 (9.8) | 48 (10.3) | 9 (7.8) | 0.439 |
| Previous stroke/TIA, n (%) | 58 (10.0) | 48 (10.3) | 10 (8.7) | 0.593 |
| Previous myocardial infarction / coronary revascularization, n (%) | 126 (21.8) | 103 (22.2) | 23 (20.0) | 0.705 |
| Previous myocardial infarction / coronary revascularization on beta blockers, n (%) | 61 (10.5) | 53 (11.4) | 8 (7.0) | 0.163 |
| Heart failure, n (%) | 83 (14.3) | 64 (13.8) | 19 (16.5) | 0.459 |
| Heart failure on ACE-I, n (%) | 25 (4.3) | 18 (3.9) | 7 (6.1) | 0.297 |
| $CHA_2DS_2$-VASc score, mean (SD) | 3.2 (1.4) | 3.1 (1.3) | 3.7 (1.5) | 0.021 |
| TiTR %[a], mean (SD) | 64.4 (19.8) | 65.0 (19.9) | 62.1 (19.5) | 0.210 |
| PCSK9, pg/ml, median (IQR) | 1200 (800–1850) | 1200 (800–1900) | 1200 (900–2000) | 0.219 |
| Fasting blood glucose, mg/dl, mean (SD) | 104.0 (28.6) | 101.6 (25.9) | 113.9 (36.1) | <0.001 |
| Creatinine, mgdl[b], mean (SD) | 1.02 (0.33) | 1.01 (0.34) | 1.04 (0.28) | 0.433 |
| eGFR (sMDRD), ml/min/1.73 cm$^{2a}$, mean (SD) | 75.1 (22.6) | 74.9 (22.6) | 75.8 (22.8) | 0.741 |
| HDL-C, mg/dl, mean (SD) | 47.6 (13.2) | 48.1 (13.1) | 45.3 (13.3) | 0.052 |
| Triglycerides, mg/dl, median (IQR) | 103 (86–135) | 101.5 (86–133) | 110 (82–134) | 0.794 |
| LDL-C, mg/dl, mean (SD) | 107.6 (30.4) | 108.6 (30.0) | 103.5 (31.9) | 0.123 |
| Therapy | | | | |
| Antiplatelets, n (%) | 104 (18.0) | 84 (18.1) | 20 (17.4) | 0.859 |
| Proton pump inhibitor, n (%) | 290 (50.8) | 223 (48.7) | 67 (59.3) | 0.046 |
| Statins, n (%) | 268 (46.2) | 213 (45.9) | 55 (47.8) | 0.726 |
| Amiodarone, n (%) | 148 (25.6) | 121 (26.1) | 27 (23.5) | 0.633 |

| | | | | |
|---|---|---|---|---|
| Digoxin, n (%) | 89 (15.4) | 72 (15.5) | 17 (14.8) | 1.000 |
| Verapamil, n (%) | 72 (12.4) | 58 (12.5) | 14 (12.2) | 0.924 |
| ACE-I/ARB, n (%) | 406 (70.1) | 318 (68.5) | 88 (76.5) | 0.111 |
| Beta-blockers, n (%) | 253 (43.7) | 204 (44.0) | 49 (42.6) | 0.834 |
| Oral antidiabetic drugs, n (%) | 81 (14.0) | 51 (11.0) | 30 (26.1) | <0.001 |
| Insulin, n (%) | 19 (3.3) | 12 (2.6) | 7 (6.1) | 0.076 |

[a]Data on 506 patients. [b]Data on 552 patients.

Abbreviations: AF, atrial fibrillation; ACE-I, angiotensin converting enzyme inhibitors; ARB, angiotensin receptor blockers; BMI, body mass index; PCSK9, proprotein convertase subtilisin/kexin type 9; T2DM, type 2 diabetes mellitus; TIA, transient ischemic attack; TiTR, time in therapeutic range

**Table 2. Stepwise logistic regression analysis of factors associated with ankle brachial index (ABI) ≥1.4**

| | **Odds ratio** | **95% confidence interval** | ***P*-value** |
|---|---|---|---|
| PCSK9 >1150 pg/ml | 1.649 | (1.047–2.598) | 0.031 |
| Female sex | 0.409 | (0.250–0.669) | <0.001 |
| T2DM | 2.768 | (1.667–4.597) | <0.001 |
| Proton pump inhibitors | 1.778 | (1.075–2.943) | 0.025 |

See methods for the list of covariates.

Abbreviations: see Table 1

**Table 3. Multivariable Cox regression analysis model of factors associated with all-cause mortality**

| | Hazard ratio | 95% CI | *P*-value |
|---|---|---|---|
| **ABI ≥1.4** | 1.626 | (1.024–2.582) | 0.039 |
| **Smoking** | 1.429 | (0.723–2.825) | 0.304 |
| **Antiplatelet** | 1.775 | (1.153–2.733) | 0.009 |
| **ACE Inhibitors/ Sartans** | 0.755 | (0.483–1.180) | 0.218 |
| **β blockers** | 0.869 | (0.561–1.347) | 0.531 |
| **Digoxin** | 0.999 | (0.598–1.670) | 0.998 |
| **Verapamil** | 0.949 | (0.543–1.658) | 0.853 |
| **Amiodarone** | 0.989 | (0.612–1.600) | 0.965 |
| **Statin** | 0.800 | (0.530–1.207) | 0.288 |
| **$CHA_2DS_2VASc$ score** | 1.249 | (1.088–1.434) | 0.002 |
| **PPI** | 1.291 | (0.858–1.943) | 0.221 |
| **Persistent/permanent AF** | 1.401 | (0.919–2.135) | 0.117 |
| **PCSK9 >2060 pg/ml** | 2.200 | (1.437–3.369) | <0.001 |

Abbreviations: ABI, ankle brachial index; AF, atrial fibrillation; PPI, proton pump inhibitors; PCSK9, proprotein convertase subtilisin/kexin type 9

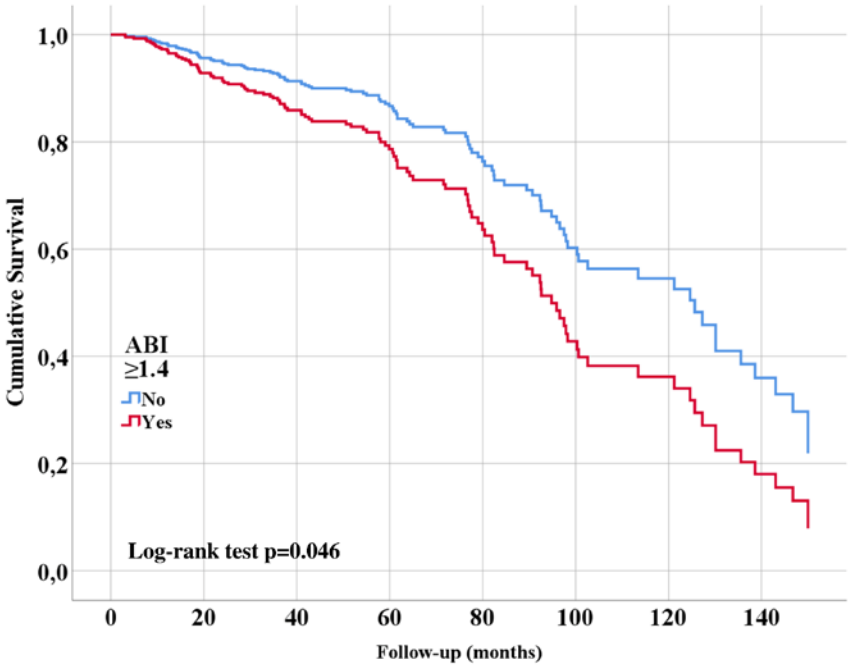


**Figure 1.** Survival curve from multivariable Cox proportional hazard regression analysis showing a direct association between ankle brachial index (ABI) ≥1.4 and all-cause mortality