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Is there an interplay between adherence to Mediterranean diet, antioxidant status and vascular disease in atrial fibrillation patients?

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Running title: Mediterranean Diet and glutathione peroxidase 3

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Abstract

Mediterranean Diet (Med-Diet) is associated with reduced incidence of vascular events (VEs) in atrial fibrillation (AF), but the mechanism accounting for its beneficial effect is only partially known. We hypothesized that Med-Diet may reduce VEs by improving antioxidant status, as assessed by glutathione peroxidase 3 (GPx3) and superoxide dismutase (SOD). We performed a prospective cohort study investigating the relationship between adherence to Med-Diet, serum baseline GPx3 and SOD activities, and the occurrence of vascular events (VEs) in 690 AF patients. GPx3 activity was directly associated with Med-Diet score (B=0.192, p<0.001) and inversely with age (B=-0.124, p=0.001), after adjustment for potential confounders; Med-Diet weakly affected SOD levels. During a mean follow-up of 46.1±28.2 months, 89 VEs were recorded; patients with VEs had lower GPx3 levels compared to those without (p=0.002); no differences regarding SOD activity were found. Multivariable Cox regression analysis showed that age (Hazard ratio [HR]:1.065, p<0.001), logGPx3 (above median, HR:0.629, p<0.05) and Med-Diet score (HR:0.547, p<0.05) predicted VEs. Med-Diet favourably modulates antioxidant activity of GPx3 in AF resulting in reduced VEs rate. We hypothesize that modulating GPx3 levels by Med-Diet could represent an additional nutritional strategy to prevent VEs in AF patients.

Introduction

Atrial Fibrillation (AF) patients disclose several atherosclerotic risk factors that may complicate their clinical course, by increasing the rate of thrombotic complications, such as ischemic stroke and myocardial infarction. Oxidative stress plays a key role in this setting, as it is implicated not only in promoting and maintaining AF but also in increasing the risk for cardiovascular events in AF patients. Another piece of information in this field has been provided by a recent study, in which we demonstrated that the activity of NADPH oxidase, an enzymatic system generating reactive oxygen species (ROS), is predictive of cardiovascular events in this setting(5).

The role of Mediterranean Diet (Med-Diet) in reducing the incidence of cardiovascular events is well established; thus, large prospective cohort studies investigated the relationship between the adherence to Med-Diet and incidence of cardiovascular events, and demonstrated that Med-Diet was effective both in primary and secondary prevention.

So far, the cardio protective property of Med-Diet has been mainly attributed to its ability to prevent, or improve, cardio-metabolic disease such as diabetes, obesity and dyslipidaemia. More recently, Med-Diet has been shown to be able to modulate oxidative stress, via reduction of Nox2-derived oxidative stress. The human body possesses natural antioxidants that maintain the redox status by counteracting the excess of ROS. Among them, the glutathione peroxidase (GPx) family plays an important role by catabolizing hydrogen peroxide, which is an oxidant species with pro-aggregating property. In addition to GPx, the thioredoxin system is emerging as another major buffer of H_2O_2, by preventing unremitted H_2O_2 emission from mitochondria in the heart tissue(9).

Recent study from our group demonstrated an inverse relationship between GPx3, the soluble isoform of GPx family, and cardiovascular events in a population with AF(6). We speculated that Med-Diet might positively influence vascular outcomes via up-regulation of GPx3. Aim of the study was to investigate the relationship between adherence to Med-Diet and circulating GPx3 in a population with AF.

Results

Among the entire cohort, 30 patients were excluded and 690 entered the study. Table 1 reports characteristics of patients. Overall, median Med-Diet score was 5.0 [4.0-6.0] points. In particular, olive oil consumption was present in 92.0%, fruit in 88.1%, vegetable/salad 68.4%, combined fruit and vegetable in 63.3%, legumes in 37.7%, fish in 14.5%, wine in 36.5%, low meat in 74.5% and

low bread/rice 34.9%. Median GPx3 activity was 21.0 [12.0-34.0] U/ml (logGPx3 2.99±0.7), median SOD was 2.1 [1.3-3.2] U/ml (logSOD 0.77±0.5).

GPx3, SOD and Med-Diet. Med-Diet score was significantly correlated with logGPx3 (r=0.191 p<0.001). A significant difference across tertiles of Med-Diet score was observed for mean values of logGPx3 levels (Fig.1, p <0.001; 1st vs. 3rd tertile of Med-Diet score, p<0.001, 2nd vs. 3rd tertile of Med-Diet score, p=0.017). In a multivariable stepwise linear regression analysis, Med-Diet score (B=0.192, p<0.001) and inversely with age (B=-0.124, p=0.001) were directly associated with logGPx3, after adjustment for potential confounders (see Methods). Using single foods as covariates (with the exception of the item 4 of combined use of fruit and vegetable), we found that vegetable/salad (B=0.130, p<0.001), fruit (B: 0.080, p=0.037) and wine (B=0.076, p=0.041) were directly associated with logGPx3 levels, while age remained inversely associated (B=-0.128, p=0.001).

SOD levels were correlated with Med-Diet adherence score (r=0.077, p=0.044). At the multivariable stepwise linear regression analysis a direct association between levels of logSOD and Med-Diet score (B=0.077, p=0.042) was found, while arterial hypertension (B=-0.075, p=0.048) was inversely associated. The same analysis using logSOD as dependent variable, showed that fish (B=0.081, p=0.033), and arterial hypertension (B=-0.075, p=0.049) were independently associated with SOD levels.

Antioxidant enzymes, VEs and Med-Diet. During a mean follow-up of 46.1±28.2 months, 89 patients experienced VEs: 22 ischemic strokes, 18 cardiac revascularizations, 25 MIs, 3 VTE, 17 cardiovascular deaths and 4 systemic embolisms.

Compared to those without, patients with VEs had significantly lower GPx3 levels [14.0 (10.0-25.0) vs. 21.0 (12.0-36.0), p=0.002]. Patients with MI/cardiac revascularization and cerebrovascular events had similar GPx3 values [12.0 (9.0-25.0) vs. 12.0 (7.5-21.0), p=0.500]; patients with cardiovascular death tended to have slightly higher GPx3 levels [18.0 (10.0-25.0), vs. cerebral and cardiac events 12.0 (18.0-24.0), p=0.312].

No differences regarding SOD activity were found (p=0.747); thus, no further survival analysis was performed with this enzyme.

A Kaplan-Meier analysis showed a significant predictive value of low logGPx3 activity (below median) for the onset of VEs at follow-up (Figure 1. Log-rank test p=0.012).

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Med-Diet score was significantly lower in patients with VEs compared to those without (p=0.024). At univariate Cox regression analysis we observed a significant risk reduction of VEs for each point of Med-Diet adherence score (HR: 0.858, 95%CI 0.748-0.985, p=0.029), and for median value of logGPx3 activity (HR: 0.581 95%CI 0.377-0.895, p=0.014).

In a multivariable Cox regression analysis, age, logGPx3 and Med-Diet score independently predicted VEs (Table 2).

Discussion

The study demonstrated that GPx3 levels are favourably influenced by adherence to Med-Diet. Previous investigations on the relationship between Med-Diet and GPx3 levels reported controversial results. Thus, an analysis conducted in 372 subjects enrolled in the PREDIMED study evidenced a lack of correlation between the interventional study with a traditional Med-Diet and serum GPx activity (3). However, laboratory analysis was restricted to total GPx activity without discriminating among GPx isoforms.

On the other hand, a positive correlation between adherence to the Med-Diet and reduced levels of GPx activity was found in an observational study on 149 twins by Day and colleagues (2). The Authors performed an intriguing study including twins so excluding early environmental or genetic confounding factors potentially affecting GPx activity. Nevertheless, this study is difficult to be translated into the general population, as it was performed only in male young subjects and investigated only total GPx activity.

In our study, the association between Med-Diet adherence and GPx3 was evidenced on a real high-risk population, as it is the one represented by AF patients. Thus, patients presented a cluster of different risk factors with a high prevalence of hypertension, previous cardiovascular or cerebrovascular events. Moreover, we included patients of both genders.

It is also clinically relevant that our study analysed a specific GPx isoform, namely GPx3, that seems to be associated to the occurrence of platelet-dependent thrombotic events as evidenced in animal and human models of Gpx3 deficiency(4). Previous study from our groups demonstrated, in fact, a key role for H_20_2 in eliciting platelet activation via thromboxane A2 over-production(7). As GPx3 degrades H_20_2, its reduced activity predisposes to platelet activation and eventually thrombosis. In accordance with this hypothesis, we have previously demonstrated an inverse association between GPx3 activity and thromboxane biosynthesis(6).

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In this context, Med-Diet, which does not interfere with anticoagulation stability, may be a suitable tool to enhance GpX3 activity in AF patients. The exact mechanism for this association is difficult to be deduced from an observational study; it is possible that some specific components of Med-Diet, such as resveratrol, may play a role via up-regulation of the mRNA expression of SOD and GPx but other components cannot be excluded(8). Thus, Med-Diet contains nutrients rich of antioxidants such as vitamin C and E, carotenoids, and flavonoids, which may contribute to the increase of GPx3 activity.

To further explore the relationship between Med-diet and antioxidants, we also investigated the relationship between adherence to Med-Diet and SOD activity, which has been shown to be associated to an increased rate of cardiovascular events in AF(6). However, though significant, the relationship between adherence to Med-Died and plasma SOD was weak.

Previous study evaluated the relationship between GPx isoforms and cardiovascular events. Thus, a prospective study involving 636 patients with suspected coronary heart disease showed an inverse relationship between the activity of the enzyme and the risk of cardiovascular events in a follow-up period of 4.7 years(1). We also investigated the interplay between GPx3 and VEs. Thus, after dividing the cohort into median value of GPx3 levels, we found that patients below the median activity of GPx3 were at higher risk of VEs in a long-term follow-up. Altogether, these data suggest that GPx isoforms may play a key role in the onset and progression of cardiovascular disease. Interestingly, we observed a significant risk reduction of VEs for each point of Med-Diet score adherence and for median value of logGPx3 activity suggesting a role for Med-Diet in protecting from VEs by influencing GPx3 levels.

The study has implications and limitations. A clinical implication of the study is that, no effective therapeutic options have been described so far to modulate antioxidant activity of GPx3 either in primary or in secondary prevention. For instance, clinical trials with selenium supplements, a cofactor of GPx, failed to induce any increase in GPx. The increased level of GPx3 associated to the high adherence to this dietary regimen suggests that nutrients from Med-Diet may favourably affect GPx3 activity; an interventional study with specific component of Med-Diet is necessary to support this finding. Moreover this study is performed on high-risk patients mostly on primary prevention thereby the results cannot be extrapolated to patients with established coronary heart disease.

In conclusion, the results from the present study support the evidence of a positive effect of Med-Diet in increasing GPx3 resulting in reduced VEs rate. Modulating GPx3 levels by Med-Diet could represent an additional nutritional strategy to prevent VEs in AF patients.

Innovation

Adherence to Med-Diet diet is associated to a lowered oxidative stress, but it is unknown if it may influence the activity of some antioxidant systems, such as GPx3 and SOD. Here we found that adherence to Med-Diet is associated to higher GPx3 activity, and that low GPx3 levels predict CVEs in high-risk patients such as those affected by atrial fibrillation. The results from this study support the hypothesis that Med-Diet could represent an additional nutritional strategy to prevent CVEs in AF patients via GPx3 activity up-regulation.

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NOTES

Methods

We performed a prospective, single-center, observational cohort study, including 720 consecutive non-valvular AF patients from I Medical Clinic - Atherothrombosis Center of Sapienza University of Rome from February 2008 to December 2013. All patients with non-valvular AF, aged >18 years of both sexes were included in the study. All patients were treated with vitamin k antagonists, after appropriate risk stratification. We excluded patients with mechanical or biologic prosthetic valves, severe valvulopathies or history of valvulopasty, congenital heart diseases, severe cognitive impairment (e.g Alzheimer's disease, Parkinson's disease) and chronic inflammatory diseases. We also excluded subjects with evidence of neoplastic diseases, liver cirrhosis, or if they were taking any vitamin or antioxidant supplementation. At baseline, for each patient anthropometric data were collected as well as data regarding concomitant diseases and treatments. Arterial hypertension, diabetes mellitus, and heart failure were classified according to international definitions(6). Written informed consent was obtained from all patients. The study was approved by the local Ethical Committee and was conducted in accordance with the principles embodied in the Declaration of Helsinki.

Analysis of adherence to Mediterranean Diet

Adherence to the Med-Diet was assessed by a short dietary questionnaire, which investigated the consumption of 9 cardio-protective foods including olive oil, fruit, vegetables or salad, legumes, fish, wine, meat and bread/rice, as previously reported(5).

Blood sampling

Blood samples were obtained during the enrolment visit. Samples were taken without stasis from patients who had fasted for at least 12 h from 7.00 to 8.00 am in serum specific tubes. The tubes were incubated for 1 h at 37°C, centrifuged to separate serum, and then frozen at -80°C until assayed.

Analysis of GPx3 and SOD activity

GPx3 activity was measured in serum by Assay Kit (Abcam). In this assay, glutathione peroxidase reduces the probe Cumene Hydroperoxide while converting reduced glutathione to its oxidized form, glutathione disulfide. The generated oxidized glutathione is converted into reduced glutathione by glutathione reductase, using nicotinamide adenine dinucleotide phosphate as reducing agent. GPx3 activity is proportional to the decrease in absorbance of nicotinamide

adenine dinucleotide phosphate at 340 nm. One unit of Glutathione peroxidase is defined as the amount of enzyme that will cause the oxidation of 1 nmole of nicotinamide adenine dinucleotide phosphate (from NADPH to $NADP^+$) per minute at 25°C. Glutathione peroxidase activity was expressed as U/ml. Intra- and inter- assays were 4.0% and 6.0%, respectively. Coefficient of variation was <10%.

We measured SOD activity by a colorimetric activity kit (Arbor Assay) in serum samples; thus, the extracellular SOD (SOD3) was measured. Samples were incubated with the substrate followed by xanthine oxidase reagent. The xanthine oxidase generates superoxide in the presence of oxygen, which converts a colourless substrate into a coloured product. The coloured product was read at 450 nm. SOD activity was expressed as U/ml. Intra- and inter-assays were 4.6% and 6.1%, respectively.

Definition of vascular events (VEs)

The primary outcome of the study was a composite endpoint of VEs. They included fatal and nonfatal ischemic stroke, fatal and nonfatal MI, cardiac or peripheral artery revascularization/coronary artery bypass surgery, cardiovascular death and venous thromboembolism (VTE). Diagnosis of myocardial Infarction (MI) was made according to the international definition as previously reported(6). The occurrence of ischemic stroke was diagnosed by motor deficits and confirmed by radiological findings. If a patient died within 4 weeks of stroke or MI, this event was recorded as fatal stroke or fatal MI. Systemic embolism was defined as an acute occlusion of a vessel of an extremity or organ, documented by imaging, surgery, or autopsy findings. Cardiovascular death included sudden death; progressive congestive heart failure; procedure related death (surgical or percutaneous revascularization); and presumed cardiovascular deaths (i.e. those for which a non-cardiovascular cause had not been clearly established). The diagnosis of VTE was confirmed by radiology or ultrasonography reports. Only the first event that occurred during follow-up was used in the analysis.

Statistical analysis

Normal distribution of parameters was assessed by Kolmogorov–Smirnov test. Categorical variables were reported as counts (percentage) and continuous variables as means +/- standard deviation (SD) or median and interquartile range. Independence of categorical variables was tested by $\chi 2$ test. Student unpaired t test and Pearson product-moment correlation analysis were

used for normally distributed continuous variables. Appropriate nonparametric tests (Mann-Whitney U test and Spearman rank correlation test) were employed for all the other variables. Group comparisons were performed using Fisher's F-test (ANOVA) or Kruskall-Wallis test when needed.

For the stepwise multivariable linear regression analysis analyses, we used different regression model for logGPx3 and SOD values, adjusting for gender, body mass index, history of MI/cardiac revascularization, Med-Diet score, smoking, arterial hypertension, diabetes, antiplatelet. To investigate the association of single foods with antioxidant enzymes activity, we repeated the regression analyses entering single items of dietary questionnaire instead of Med-Diet adherence score as covariate.

After dividing the AF population according to the median value of logGPx3, the cumulative incidence was estimated using a Kaplan–Meier product-limit estimator. Survival curves were formally compared using the log-rank test. Cox proportional hazards analysis was used to calculate the adjusted relative hazards of VEs by each clinical variable. The multivariable analyses were performed including the same variables specified above for linear regression analysis.

Only p values lower than 0.05 were considered as statistically significant. All tests were two-tailed and analyses were performed using computer software packages (SPSS-18.0, SPSS Inc.).

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Authors Disclosure Statement.

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Pasquale Pignatelli: none

Abbreviations

Atrial fibrillation (AF)

Glutathione peroxidase 3 (GPx3)

Mediterranean diet (Med-Diet)

Myocardial infarction (MI)

Nicotinamide adenine dinucleotide phosphate (NADP)

Reactive oxygen species (ROS)

Superoxide dismutase (SOD)

Vascular events (VEs)

Venous thromboembolism (VTE).

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Figure Legends

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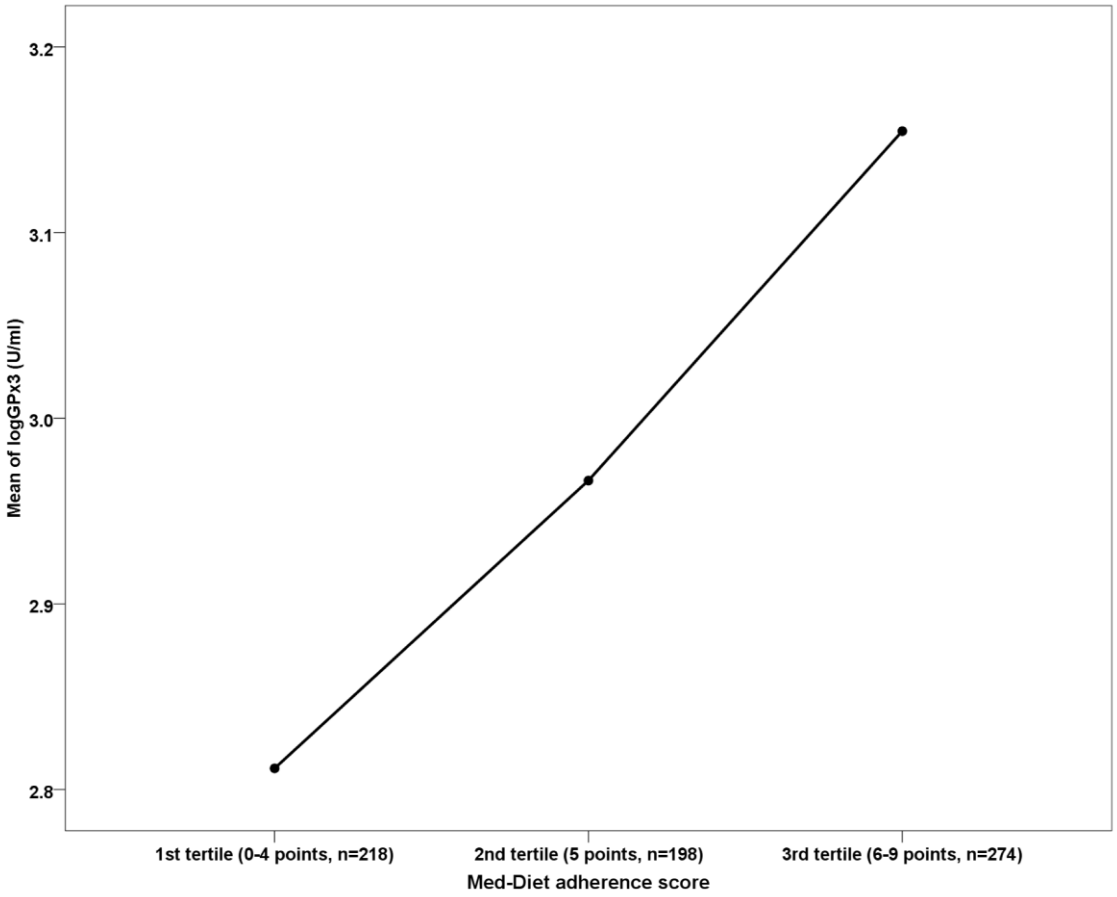

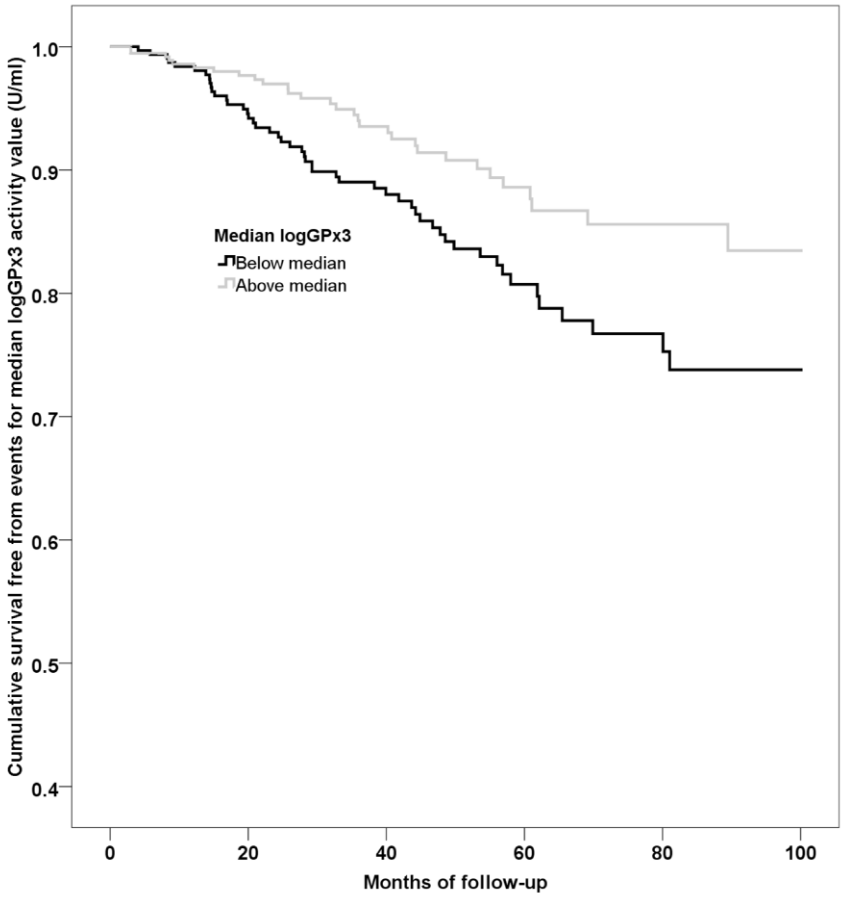

Figure 1. Values of mean logGPx3 according to the tertiles of the Mediterranean diet adherence score.

Figure 2. Kaplan-Meier survival curves according to the median value of logGPx3.

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Table 1. Characteristics of study population

	Overall (n=690)
Age *(years)*	72.7±8.0
Women *(%)*	41.6
Body Mass Index (kg/m^2)	27.6±4.8
Mediterranean Diet score[#]	5.0 [4.0-6.0]
CHA_2DS_2-VASc Score[#]	3.0 [2.0-4.0]
Arterial Hypertension (%)	94.6
Diabetes (%)	19.0
Previous cerebrovascular events (%)	12.8
Heart Failure (%)	13.6
Previous cardiovascular events (%)	20.4
Antiplatelet drugs (%)	15.2

[#]data expressed as median and interquartile range

Table 2. Multivariable Cox regression analysis of factors predicting vascular events.

	Hazard Ratio	95% Confidence Intervals	p value
Med-Diet adherence score	0.547	0.322-0.926	<0.05
logGPx3 (above median)	0.629	0.406-0.974	<0.05
Female sex	0.814	0.522-1.270	0.365
Age	1.065	1.033-1.098	<0.001
Body mass index	1.025	0.979-1.074	0.293
Arterial hypertension	0.575	0.227-1.456	0.243
Diabetes	1.517	0.930-2.475	0.095
Heart failure	1.404	0.824-2.394	0.212
Previous cerebrovascular events	1.440	0.841-2.467	0.184
Previous cardiovascular events	1.352	0.819-2.233	0.238
Antiplatelet drugs	1.356	0.792-2.320	0.267