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VNTR Polymorphism in Intron 4 of the *eNOS* Gene and the Risk of Gastrointestinal Bleeding: A Case-control Study

Marcela Forgerini[1], Gustavo Urbano[2], Tales Rubens de Nadai[3], Cleslei Fernando Zanelli[4], Sandro Roberto Valentini[5], Patrícia de Carvalho Mastroianni[6]

1) Department of Drugs and Medicines, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara;
2) Department of Surgery, School of Medicine, University of São Paulo, Ribeirão Preto;
3) Department of Public Health, Bauru School of Dentistry, University of São Paulo (USP), Bauru;
4) Department of Biological Sciences, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara;
5) Department of Biological Sciences, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara;
6) Department of Drugs and Medicines, School of Pharmaceutical Sciences, São Paulo State University (UNESP), Araraquara, Brazil

Address for correspondence:
Patricia de Carvalho Mastroianni
Associate Professor, Department of Drugs and Medicines, School of Pharmaceutical Sciences São Paulo State University (UNESP), Highway Jaú, Km 01 s / n 14800-901 Araraquara, Brazil.
patricia.mastroianni@unesp.br

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ABSTRACT

Background & Aims: Considering the lack of knowledge regarding the influence of the variable number of repeats of 27 pb in intron 4 (4b/4a VNTR - rs61722009) of the endothelial nitric oxide synthase (*eNOS*) on the drug response, we assessed the influence of this polymorphism for the risk of upper gastrointestinal bleeding (UGIB).
Methods: A case-control study, including 200 cases and 706 controls, was conducted in a Brazilian hospital complex. Cases were participants with UGIB diagnosis. Controls were participants admitted to surgical procedures not related to gastrointestinal problems. The 4b/4a VNTR was determined through polymerase chain reaction followed by fragment analysis. Conditional logistic regression models were designed. The additive interaction between the presence of the 4b/4a VNTR variant and the use of low-dose aspirin (LDA) and nonsteroidal anti-inflammatory drugs (NSAIDs) was calculated by fitting the Cox regression model through the parameters of Synergism index (S) and Relative Excess Risk Due To Interaction (RERI).
Results: The presence of the 4b/4a VNTR variant did not increase the risk of UGIB: carriers of the 4a/4a genotype (OR=0.37, 95%CI: 0.09-1.45) and of the variant allele "4a" (OR=0.91, 95%CI: 0.55-1.51). The risk of UGIB in LDA users carriers of the wild genotype (OR=4.96, 95%CI: 2.04- 2.06) and the variant allele "4a" (OR=3.49, 95%CI: 1.18-10.38) is similar, as well as for NSAID users carriers of the wild genotype (OR=5.73, 95%CI: 2.61-12.60) and variant allele "4a" (OR=5.51, 95%CI: 1.42-15.82). No additive interaction was identified between the presence of the genetic variant and the use of LDA [RERI: -1.44 (95%CI: -6.02–3.14; S: 0.63 (95%CI: -1.97–1.15)] and NSAIDs [RERI: -0.13 (95%CI: -6.79–6.53; S: 0.97 (95%CI: -0.23–4.19)] on the UGIB risk.
Conclusion: Our data suggests that there is no increase in the magnitude of UGIB risk in LDA and NSAIDs users' carrying the variant allele "4a".

Key words: gastrointestinal bleeding – UGIB – peptic ulcer hemorrhage – aspirin – nonsteroidal anti-inflammatory drugs – NSAIDs – VNTR Sequences; tandem repeat sequences.

Abbreviations: CI: confidence interval; *eNOS*: endothelial nitric oxide synthase; *H. pylori: Helicobacter pylori*; LDA: low dose aspirin; NO: nitric oxide; NSAID: nonsteroidal anti-inflammatory drug; RERI: relative excess risk due to interaction; S: synergism index; UDE: upper digestive endoscopy; UGIB: upper gastrointestinal bleeding.

INTRODUCTION

The clinical implications of the single nucleotide polymorphisms rs2070744 (−786T>C in the promoter region) and rs1799983 (894G>T, Glu298Asp) and the variable number of repeats of 27 pb in intron 4 (4b/4a VNTR - rs61722009) of the endothelial nitric oxide synthase (*eNOS*) in several health problems have been studied (i.e., cardiovascular diseases and diabetes mellitus) [1-3]. These variants are located in an important region of the *NOS3* gene and they possibly affect the production of nitric oxide (NO) and physiological processes [4]. However, there is a lack of knowledge with regard to the influence of these variants on the drug response, especially in users of low-dose aspirin (LDA) and nonsteroidal anti-inflammatory drugs (NSAIDs) [5].

In our previous study, we evaluated in the Brazilian population the influence of rs2070744 and rs1799983 variants on the risk of upper gastrointestinal bleeding (UGIB) [6], a potential adverse drug reaction associated with high mortality, hospital readmissions, and the need for surgical intervention [7]. When the subgroups of LDA and NSAIDs

users were considered, carriers of the variant alleles of rs2070744 and rs1799983 had a higher risk of developing UGIB [6], corroborating with a recent case-control study conducted with the Spanish population that also identified an increased risk of UGIB in LDA users carrying the variant allele of rs1799983 [8].

In this context, in addition to these two single nucleotide polymorphisms, we also identified through a systematic review three Spanish studies reporting the influence of the 4b/4a VNTR on the risk of UGIB [9].

The first investigation was a case-control study conducted by Serrano et al. [10] in which 188 participants with peptic disease, 86 of them with UGIB, and 120 controls were included. The authors identified a reduced risk of UGIB in carriers of the variant allele "4a" (OR=0.49, 95%CI: 0.25-0.95) [10] corroborating with the findings of Piazuelo et al. [11] which included 89 LDA users diagnosed with UGIB and 266 controls (two control groups: LDA and non-LDA users) (OR=0.39, 95%CI: 0.18-0.85). Both studies were conducted by the same research group and included only Caucasian participants. However, in the first study the process of recruitment and matching of participants was unclear, and it was not reported whether statistical analysis adjustment was performed considering confounding variables [9]. Such aspects might prove relevant methodological limitations.

Finally, the recently published case-control study conducted by Mallah et al. [12] included 335 participants with UGIB and 744 controls, and the recruitment was restricted to participants of European origin and regardless of LDA/NSAIDs use. The authors did not identify a difference in the magnitude of the risk of UGIB between wild participants and carriers of the "4a" variant allele nor additive interaction between the presence of the allele variant and the use of LDA or NSAIDs on UGIB risk.

Considering that the three studies evaluated the influence of the 4b/4a VNTR variant on the risk of UGIB in Spanish Caucasians and the difficulty in extrapolating the findings to the Brazilian population due to the high miscegenation, it was intended to assess the influence of this variant on the risk of UGIB in our population.

METHODS

Ethical Aspects

The Research Ethics Committee of the São Paulo State University (protocol number 1,657,615) and the Clinical Hospital of the Ribeirão Preto Medical School of the University of São Paulo (protocol number 1,536,886) approved this study. All participants signed the informed consent before their enrollment in this study.

Study Design and Setting

A case-control study including 200 cases and 706 controls, matched by gender and age (± 5 years), was conducted during July 2016 to March 2020 in the hospital complex of Clinical Hospital of the Ribeirão Preto Medical School of the University of São Paulo, Brazil, which comprises four hospitals.

The report of this study was based on the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE).

Participants

The case group comprised 200 patients diagnosed with UGIB of non-variceal etiology confirmed by upper digestive endoscopy (UDE) or surgical intervention. In addition to the endoscopic findings of interest [ulcer (gastric, duodenal, or pyloric); erosions or acute lesions of the gastric or duodenal mucosa; and erosive gastritis or duodenitis, with or without active or recent bleeding], clinical signs and symptoms such as hematemesis, dark vomiting or in coffee grounds, melena and/or hematochezia were considered. Patients who did not undergo UDE within the first 48 hours of hospital admission, with endoscopic findings of neoplasia, Mallory-Weiss tears, and Dieulafoy lesions, with in-hospital UGIB, with severe health status, and who were hospitalized within 15 days prior to the current hospital admission were excluded.

Daily, three researchers (M.F., G.U. and T.R.N.) assessed the endoscopic reports of patients hospitalized and submitted to UDE in the hospital complex under study, in order to identify those diagnosed with UGIB of non-variceal etiology.

During 46 months of screening, 2,883 UDE were evaluated (2,557 patients) and the mean number of diagnoses of UGIB was four per month (SD: ± 3). Ninety percent of the cases, among other endoscopic findings, had peptic ulcer (gastric or duodenal), and most of them were classified as Forrest IIa (non-bleeding visible vessel) and Forrest III (lesion without bleeding, clean spot).

The control group included 706 patients who underwent surgery not related to gastrointestinal disorders (i.e., phakectomy, inguinal/umbilical hernia correction, and prostatectomy).

Participants under 18 years of age, history of neoplasia, coagulopathies, immunodeficiency syndrome, and cirrhosis, nasogastric or percutaneous tube holders, narcotics users, and non-residents of the study region for at least three months were excluded.

Both participants were recruited from the same hospital complex and the inclusion and exclusion criteria for the participants of case and control groups are described in detail in our two previous studies [6, 13].

Data Collection

The recruitment of participants and data collection was performed by four previously trained researchers (M.F., G.U., T.R.N. and P.C.M.) using a previously designed and validated questionnaire [14]. In addition to the interview with the patient and/or family member, the electronic medical records of the hospital complex, medical prescriptions, and laboratory tests were consulted. After the interview, the venous blood (5 mL) was collected in ethylenediamine tetra-acetic acid from all participants.

The following data were recorded: gender, age, self-declared ethnicity (white, black, brown, and oriental), schooling, body mass index, previous personal history of gastrointestinal disorders (ulcer, dyspepsia, and bleeding), *H. pylori* infection, comorbidities (high blood pressure, diabetes mellitus, dyslipidemia, arthritis, and arthrosis), drug therapy in use (LDA, other antiplatelet agents, NSAIDs, oral anticoagulants, and proton pump inhibitors), and lifestyle habits (smoking, alcohol, and coffee consumption). The anticoagulants used by

the participants consisted of warfarin, apixaban, rivaroxaban, and phenprocoumon.

To assess the drug therapy in use, an etiological window of seven days before the index day was considered, which is defined as the day on which signs and symptoms of UGIB started (cases) and the date of the interview (controls) [15]. The use of LDA was defined as the continuous use of aspirin in the indication of prevention of cardiovascular events in doses lower than 150 milligrams and for at least three months.

For each participant, a pharmacotherapeutic anamnesis was performed considering the drug therapy in use, dose, pharmaceutical form, and time of use, in addition to evaluate the defined daily dose of each medicine in use. No participant in the case group overdosed on antiplatelet agents, NSAIDs, and/or anticoagulants.

Regarding lifestyle habits, the consumption of alcohol, tobacco and coffee was recorded in the two months preceding the index date. Alcohol consumption was stratified according to grams of alcohol consumed (zero; > 0 to ≤ 30 grams of alcohol/day and > 30 grams of alcohol/day), while tobacco consumption was stratified according to the number of cigarettes consumed per day (zero; 1 to 15 cigarettes/day; > 15 cigarettes/day). Coffee consumption was stratified in zero; > 0 mL ≤ 100; > 100 > mL ≤ 200; > 200 mL ≤ 500; and > 500 mL.

All data obtained in this study, as well as the results of genotyping and *H. pylori* infection, were entered into the Research Electronic Data Capture (RedCap) database.

Helicobacter Pylori Infection

The presence of *H. pylori* infection was determined through the determination of IgG antibodies in the plasma samples using the chemiluminescence technique. The test results of *H. pylori* infection were stratified into reagent (≥ 1.10 U IgG/mL), non-reagent (≤ 0.90 U IgG/mL) and inconclusive (0.91 to 1.09 U IgG/mL).

Genotyping

Genomic DNA was extracted from the venous blood samples using the Maxwell® 16 Blood DNA Purification Kit (Promega, Madison, WI, USA). DNA concentration and purity were determined using Invitrogen Qubit 4 Fluorometer and using the Qubit ™ dsDNA HS Assay kit (Applied Biosystems, Foster City, USA).

The 4b/4a VNTR was determined by polymerase chain reaction (PCR) followed by fragment analysis using the primer pairs proposed by Wang and colleagues (1997) [16]: 5'-AGGCCCTATGGTAGTGCCTTT-3' (forward – fluorescence-labeled FAM) and 5'-TCTCTTAGTGCTGGTCAC-3' (reverse) (AppliedBiosystems, Foster City, USA).

A 10 µL volume was used for each PCR reaction containing: 6.0 µL ultrapure water; 2.0 µL genomic DNA (10 ng/µL); 1.0 µL 10 x PCR buffer (Invitrogen); 0.3 µL magnesium chloride (MgCl2, 50 mM) (Invitrogen); 0.2 µL of deoxynucleotide triphosphates (dNTP, 10 mM) (AppliedBiosystems, Foster City, USA); 0.2 µL of each primer (25 mM); and 0.1 µL of 5U/µL Taq polymerase (Invitrogen).

The PCR conditions were performed with the following protocol: 95°C for five minutes; 35 cycles of 95°C for 30 seconds, 56°C for 40 seconds and 72°C for one minute and 30 seconds; and final extension at 72°C for 10 minutes. The amplification product was analyzed in capillary electrophoresis using the ABI 3130 Genetic Analyzer (AppliedBiosystems, Foster City, USA).

To prepare the sample for analysis in capillary electrophoresis, a reaction volume of 10 µL was standardized: 8.75 µL Hi-Di™ Formamide (Applied Biosystems), 0.25 µL of Ladder GeneScan™ 1000ROX™ (AppliedBiosystems, Foster City, USA), and 1.00 µL of PCR product.

The presence of a fragment of 420 base pairs represented the wild-type genotype (4b/4b; 5 copies of the 27 bp repeat); two fragments of 393 and 420 base pairs indicated a heterozygous genotype (4b/4a); and a 393 base pair fragment represented a homozygous genotype for the variant allele (4a/4a, 4 copies of the 27 bp repeat). For quality control, 10% of the samples were randomly selected to repeat the analyses (91 DNA samples). Capillary electrophoresis results were added to the Data connect cloud (Applied Biosystems) for analysis of fragment plots.

Statistical Analysis

Hardy–Weinberg Equilibrium

Genotyping quality and possible bias in the selection of participants of the control group were verified using the Hardy–Weinberg Equilibrium test using the BioEstat 5.0 program.

Regression Models

The outcome variable was defined as UGIB, this being a dichotomous variable (case and control), and the independent variable was defined as 4b/4a VNTR.

The association between 4b/4a VNTR variant alleles and the risk to develop UGIB was estimated by odds ratio (OR) adopting a level of statistical significance of 0.05 for decision making and building 95% confidence interval (95%CI) using conditional logistic regression models.

Conditional logistic regression models were constructed using COX regression survival analysis in the SPSS v.26 software (IBM Company, Chicago, IL, USA) and the strata of cases and their respective controls were considered.

Firstly, the confounding variables and the outcome variable (UGIB) were considered under a bivariate analysis to select the values to compose the conditional logistic regression models. For qualitative variables the Chi-square test or Fisher's exact test was performed, and for quantitative variables the Student's *t*-test.

In this analysis, the following variables were evaluated: self-declared ethnicity, schooling, body mass index, previous personal history of gastrointestinal disorders, *H. pylori* infection, comorbidities, drug therapy in use, and lifestyle habits. The variables gender and age were not included in the model, since they were controlled in the study planning by pairing the participants of the case and control groups.

The conditional logistic regression model was built with all the confounding variables selected in the bivariate analysis (p-value ≤ 0.20) concomitantly (insert method of SPSS Program). For the dichotomous variables, the reference category was represented by the category "no" and for the 4b/4a VNTR variant, the wild genotype was considered the reference category (4b/4b genotype).

Multiplicative and Additive Scale Interaction

The multiplicative interaction between the presence of the 4b/4a VNTR variant and the use of LDA and NSAIDs was calculated by fitting the Cox regression model.

The additive interaction between the presence of the 4b/4a VNTR variant and the use of LDA and NSAIDs was estimated employing the Synergism index (S) and Relative excess risk due to interaction (RERI) parameters [17]. The respective 95%CI was constructed according to the method proposed by Figueiras et al. [18].

For additive interaction analysis, participants were divided into four groups according to genotype and LDA/NSAIDs use status:

$RERI = OR_{11} - OR_{01} - OR_{10} + 1$

$S = (OR_{11} - 1)/((OR_{01} - 1) + (OR_{10} - 1))$

11: genetic variation and LDA/NSAIDs use

01: absence of genetic variation and no use of LDA/NSAIDs

10: genetic variation and no use of LDA/NSAIDs

Multiplicative and additive interaction analyses were adjusted for the selected confounding variables in the bivariate analysis (p-value ≤ 0.20).

Model Fit Measures

To avoid distortions of the model effect measures, the correlation matrix of regression coefficients was analyzed for potential linear relationships between the predictor variables and the outcome variable (UGIB). To evaluate model fit, the likelihood ratio test (2LL), the Akaike information criterion (AIC), and the Bayesian information criterion (BIC) were adopted.

Data Transparency

This is the first Brazilian study to assess the influence of genetic variants on the risk of UGIB and the full-methodology protocol is available at the *Open Science Framework* (OSF) [19].

RESULTS

Baseline Characteristics of Participants

Most participants were male (case group: 72.5% and control group: 72.5%), self-declared white (case group: 81.3% and control group: 84.2%), with a mean age of 60.1 (±16.3) and 59.9 (±15.8) years, respectively.

A percentage of 31.6 of the participants in the case group and 32.8% of the participants in the control group are carriers of the "4a" variant allele. The minor allele frequency observed was 0.17 for the case group and 0.18 for the control group, and this variant attended the Hardy-Weinberg equilibrium (control group: p-value 0.9881). No difference in allele distribution was identified between case and control group participants (Chi-square test: 0.117, p=0.733).

The sociodemographic and clinical characteristics of the 200 participants in the case group and 706 in the control group are described in Table I.

Association of 4b/4a VNTR and the Risk of UGIB

No statistical significance was identified between the presence of the variant allele "4a" and the risk of UGIB: carriers of the 4a/4a genotype (OR=0.37, 95%CI: 0.09-1.45) and carriers of the "4a" allele (OR=0.91, 95%CI: 0.55-1.51).

Table I. Demographic and clinical characteristics of the participants

	Cases (%) n=200	Controls (%) n=706	p[a]
Gender (male)	145 (72.5)	512 (72.5)	0.933
Age [mean (±SD)]	60.2 (16.3)	59.8 (15.8)	0.750
Ethnicity (self-declared)			0.406
White	143 (81.3)	542 (84.2)	
Brown	33 (18.8)	102 (15.8)	
Black	23 (11.5)	58 (8.2)	
Oriental	1 (0.5)	4 (0.6)	
Schooling (years)			0.237
0 – 4	105 (52.5)	370 (52.4)	
5 – 11	67 (33.5)	248 (35.1)	
> 11	28 (14.0)	88 (12.5)	
Body mass index kg/m^2	< 0.001[b]		
Underweight (<18)	10 (5.0)	15 (2.1)	
Normal (≥ 18 - ≤ 24)	80 (40.0)	197 (27.9)	
Overweight (≥ 25 - ≤ 29.9)	60 (30.0)	263 (37.3)	
Obesity I, II, and III (≥ 30)	48 (24.0)	228 (32.3)	
Missing data	2 (1.0)	3 (0.4)	
Personal history of gastrointestinal diseases			
Ulcer	44 (22.1)	64 (9.1)	< 0.001[b]
Bleeding	35 (17.6)	94 (13.3)	0.159[b]
Dyspepsia	60 (30.2)	291 (41.2)	0.006[b]
Helicobacter pylori infection	142 (76.3)	388 (57.6)	< 0.001[b]
Comorbidities			
High blood pressure	104 (52.0)	371 (52.5)	0.954
Diabetes *mellitus*	38 (19.1)	158 (22.4)	0.370
Dyslipidemia	22 (11.0)	165 (23.4)	0.001[b]
Arthrosis	9 (4.5)	48 (6.9)	0.296
Arthritis	3 (1.5)	22 (3.1)	0.364
Drug therapy in use (ATC)			
Proton pump inhibitors (A02BC)	36 (18.0)	125 (17.7)	0.993
Oral anticoagulants (B01A)	22 (11.0)	18 (2.5)	<0.001[b]
LDA (B01AC06)	51 (25.5)	90 (12.7)	<0.001[b]
Other antiplatelet agents (B01AC)	26 (13.0)	61 (8.6)	0.065[b]
NSAIDs (M01A)	43 (23.6)	71 (10.2)	<0.001[b]
Tobacco consumption			< 0.001[b]
No consumption (0 cigarette)	136 (68.0)	580 (82.2)	
Moderate consumption (1 to 15 cigarettes/day)	23 (11.5)	50 (7.0)	
Heavy consumption (> 15 cigarettes/day)	35 (17.5)	55 (7.8)	
Missing data	6 (3.0)	21 (3.0)	
Alcohol intake	< 0.001[b]		
0 gram	103 (51.5)	392 (55.5)	
0 to ≤ 30 grams of alcohol/day	71 (35.5)	297 (42.1)	
> 30 grams of alcohol/day	26 (13.0)	17 (3.3)	
Coffee consumption			< 0.001[b]
0	30 (15.0)	66 (9.3)	
0 to ≤ 100 mL	101 (50.5)	469 (66.4)	

Table I (continued)			
100 to 200 mL	24 (12.0)	57 (8.1)	
200 to ≤ 500 mL	38 (19.0)	73 (10.3)	
> 500 mL	7 (3.5)	41 (5.8)	
4b/4a VNTR			0.943
4b/4b (wild type)	132 (68.4)	473 (67.1)	
4b/4a (heterozygous)	55 (28.5)	209 (29.6)	
4a/4a (homozygous for the variant allele)	6 (3.1)	23 (3.2)	
Carriers of the "4a" allele	61 (31.6)	232 (32.8)	
Missing data	7	1	

[a] p-value is polychotomous and represents the entire variable; [b] variables with p-value ≤ 0.20 and selected for the unconditional regression models. ATC: Anatomical Therapeutic Chemical; LDA: low-dose aspirin; mL: milliliter; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; SD: standard deviation; 4b/4a VNTR: variable number of repeats of 27pb in intron 4 of the endothelial nitric oxide synthase (*eNOS*); *: statistical significance.

The risk of UGIB in LDA users carriers of the wild genotype (OR=4.96, 95%CI: 2.04-12.06) and the variant allele "4a" (OR=3.49, 95%CI: 1.18-10.38) is similar as well as for NSAIDs users carriers of the wild genotype (OR=5.73, 95%CI: 2.61-12.60) and variant allele "4a" (OR=5.51, 95%CI: 1.42-15.82). No increased magnitude of UGIB risk was observed in participants carrying the variant allele "4a".

No additive interaction was identified between the presence of the variant allele "4a" and the use of LDA/NSAIDs and the risk of UGIB. Despite the lack of statistical significance, our findings indicate a possible reduction in the odds ratio of UGIB in carriers of the variant allele "4a".

Association of Confounding Variables and the Risk of UGIB

Overweight body mass index (OR=2.90, 95%CI: 1.55-5.44); history of ulcer (OR= 4.65, 95%CI: 2.13-10. 18); *H. pylori* infection (OR=2.84, 95%CI: 1.67-4.84); LDA (OR=4.47; 95%CI: 2.01-9.94), NSAIDs (OR=6.28, 95%CI: 3.17-12. 43) and oral anticoagulants use (OR=8.30; 95%CI: 3.03-22.71); tobacco consumption [1 to 15 cigarettes/day (OR=2.68, 95%CI: 1.16-6.20) and > 15 cigarettes/day (OR=4.55, 95%CI: 2.11-9.82) and alcohol intake > 30 grams/day (OR=6.67, 95%CI: 2.50-17.85) were identified as risk factors for UGIB.

History of dyspepsia (OR=0.31, 95%CI: 0.18-0.55) and coffee consumption above 500 mL/day (OR=0.21, 95%CI: 0.05-0.91) were identified as protective factors for UGIB (Table II).

Table II. Risk of upper gastrointestinal bleeding after the conditional regression model

Variable	OR	95% CI		p
		Inferior	Superior	
Body mass index kg/m^2				
Normal (≥ 18 - ≤ 24)	1.00	-	-	-
Underweight (<18)	1.86	1.00	3.48	0.051
Overweight (≥ 25 - ≤ 29.9)	2.90	1.55	5.44	0.001*
Obesity I, II, and III (≥ 30)	1.67	0.35	8.00	0.518
History of ulcer	4.65	2.13	10.18	< 0.001*
History of bleeding	1.31	0.65	2.60	0.450
History of dyspepsia	0.31	0.18	0.55	< 0.001*
Helicobacter pylori infection	2.84	1.67	4.84	< 0.001*
Dyslipidemia	0.26	0.12	0.56	0.001*
Anticoagulants use	8.30	3.03	22.71	< 0.001*
LDA use	4.47	2.01	9.94	< 0.001*
Other antiplatelet agents use	0.80	0.30	2.09	0.643
NSAIDs use	6.28	3.17	12.43	< 0.001*
Tobacco consumption				
No consumption (0 cigarette)	1.00	-	-	-
Moderate consumption (1 to 15 cigarettes/day)	2.68	1.16	6.20	0.022*
Heavy consumption (> 15 cigarettes/day)	4.55	2.11	9.82	< 0.001*
Alcohol intake				
0 gram	1.00	-	-	-
0 to ≤ 30 grams of alcohol/day	1.02	0.61	1.73	0.928
> 30 grams of alcohol/day	6.67	2.50	17.85	< 0.001*
Coffee consumption				
mL = 0	1.00			
> 0 mL ≤ 100	0.62	0.29	1.31	0.209
> 100 > mL ≤ 200	0.98	0.36	2.66	0.966
> 200 mL ≤ 500	1.48	0.59	3.70	0.399
> 500 mL	0.21	0.05	0.93	0.040*
4b/4a VNTR				
4b/4b (wild type)	1.00	-	-	-
4b/4a (heterozygous)	1.01	0.61	1.70	0.957
4a/4a (homozygous for the variant allele)	0.37	0.09	1.45	0.153
Carriers of the „4a" allele	0.91	0.55	1.51	0.739

CI: confidence interval; LDA: low-dose aspirin; mL: milliliter; NSAIDs: non-steroidal anti-inflammatory drugs; OR: odds ratio; 4b/4a VNTR: variable number of repeats of 27pb in intron 4 of the endothelial nitric oxide synthase (*eNOS*); *: statistical significance.

DISCUSSION

Polymorphisms in genes that can potentially affect platelet-dependent clotting mechanisms and/or the platelet response to LDA are candidates for a possible association with the risk of UGIB [11]. Although variants in the *NOS3* gene might be auspicious because they possibly affect the availability of NO [4], which is a regulator of platelet function and modulator of mucosal defense and repair [20], our findings suggest that the presence of the 4b/4a VNTR variant is not associated with an increased risk of UGIB. Besides, the risk of UGIB was similar between users of LDA/NSAIDs carrying the wild genotype and those carrying the variant allele "4a".

Our findings corroborate the full case-control study conducted by Mallah et al. [12] with the Spanish population, while Serrano et al. [10] and Pizuaello et al. [11] identified that patients carrying the "4a" allele (variant allele) had a lower risk of developing UGIB.

Table III. Multiplicative and additive interaction between the presence of the 4b/4a VNTR variant and the use of low-dose aspirin and non-steroidal anti-inflammatory drugs in the risk of upper gastrointestinal bleeding

	Wild-type			Genetic variation			RERI (95% CI)	S (95% CI)
	N/N case/control	OR (95% CI)	p	N/N case/ control	OR (95% CI)	p		
LDA							-1.44 (-6.02-3.14)	0.63 (-1.97- 1.15)
No use	99/415	1.00		45/199	0.98 (0.57-1.69)	0.934		
Use	32/57	4.96 (2.04-12.06)	< 0.001*	16/33	3.49 (1.18- 10.38)	0.024*		
NSAIDs							-0.13 (-6.79-6.53)	0.97 (0.23-4.19)
No use	93/424	1.00		42/197	0.91 (0.52-1.59)	0.737		
Use	26/40	5.73 (2.61-12.60)	< 0.001*	13/31	5.51(1.92-15.82)	0.001*		

95% CI: confidence interval; LDA: low-dose aspirin; NSAIDs: Non-steroidal anti-inflammatory drugs; N: number of participants of case and control group; OR: odds ratio; RERI: Relative excess risk due to interaction; S: Synergism index; *: statistical significance.. Analysis adjusted for the following confounding variables: body mass index kg/m^2; personal history of ulcer, bleeding, and dyspepsia; *Helicobacter pylori* infection; dyslipidemia; use of oral anticoagulants, low-dose aspirin, other antiplatelet agents, and non-steroidal anti-inflammatory drugs; tobacco consumption; alcohol intake; and coffee consumption. Model fit measures of RERI and S for LDA users: -2LL: 272.55; AIC: 320.55; and BIC: 428.51. Model fit measures of RERI and S for NSAIDs users: -2LL: 272.83; AIC: 320.83; and BIC: 428.71.

The *NOS3* gene encodes the endothelial nitric oxide synthase (*eNOS*), one of the main isoenzymes responsible for the production of NO and known to be expressed in the platelets being responsible for the modulation of the platelet phenotype [21]. The VNTR of 27 bp in intron 4 of *eNOS* regulates the *NOS3* gene at a post-transcription level by the formation of a small interfering RNA (sir-RNA) [22]. Therefore, the 4b/4a VNTR variant might have a functional impact on the formation of sir-RNA that negatively regulates *eNOS* expression [22].

Endothelial cells bearing the "4b" allele have been reported to have a higher concentration of sirRNA leading to reduced levels of *eNOS* mRNA when compared with cells bearing "4a" variant allele [22, 23]. Hence, a lower *eNOS* expression will possibly lead to lower NO levels, reduced platelet aggregation process and, consequently, an increased risk of bleeding [21]. This hypothesis could justify the findings of Serrano et al. [10] and Pizuaello et al. [11].

Furthermore, the case-control study conducted by Piazuelo et al. [11] included participants using LDA, which may also justify the UGIB protection observed in carriers of the "4a" allele, since LDA use increases *eNOS* expression, NO production and bioavailability in platelets, and inhibition of platelet aggregation [24]. However, the authors did not assess additive interaction, which could elucidate whether there was a joint biological action of LDA use and the presence of the "4a" allele in reducing the risk of UGIB.

The controversial data can be justified due to differences in methodological and, especially, population aspects. It is known that ethnicity is directly related to the genomic ancestry of a population and, consequently, in the presence and frequency of genetic variants [25]. Furthermore, extensive ethnogenomic diversity was observed in genetic variants in the *NOS3* gene [26], which may be associated with possible clinical and pathophysiological implications of health problems as well as drug response.

When observing the allele frequency in Serrano's et al. [10] study, it is noted that patients with UGIB had the 4b/4b genotype more frequently than participants of the control group (OR=2.02, 95%CI: 1.04-3.94), whereas in Piazuello's et al. [11] study, patients with UGIB had a lower chance of carrying the "4a" allele when compared to control participants (22.7% vs. 35.2%). In our study, we observed no difference in allelic distribution between participants in the case and control groups (p-value: 0.733) as well as in the full case-control study of Mallah et al. [12].

Regarding the lack of additive interaction observed in our study, our findings could be justified by the small number of participants using LDA/NSAIDs (16 cases and 36 controls/13 cases and 31 controls). Therefore, it would be relevant that future studies delineate inclusion only for participants using LDA/NSAIDs in addition to haplotype analysis for improved genetic information when compared with single-locus analysis, since the combination of alleles for *NOS3* polymorphisms affects the NO formation and availability [4].

Ultimately, this study has strengths and limitations. This is the first time this type of study was performed in the more diverse Brazilian population, the cases were recruited during 46 months (a mean of four diagnoses of UGIB/month) [13], inclusion of only biologically unrelated participants in order to reduce possible biases, and data collection through face-to-face interviews including several confounding variables for UGIB. The limitations of this study are the non-conduct of the haplotype analyses of the T-786C, 4b/4a VNTR and Glu298Asp polymorphisms of *NOS3* gene and the limited number of LDA/NSAIDs users. In addition, further studies with larger sample size are required considering that low statistical power is frequent in studies that assess the influence of genetic polymorphisms [27].

CONCLUSIONS

In this exploratory study, the risk of UGIB in LDA users carriers of the wild and the variant allele "4a" is similar, as well as for NSAIDs users and no additive interaction was identified between the presence of the polymorphism and the use of LDA and NSAIDs on the UGIB risk. Hence, our data suggest that there is no increase in the magnitude of UGIB risk in users of LDA and NSAIDs carrying the variant allele "4a" of 4b/4a VNTR in intron 4 of *eNOS*.

Conflicts of interest: None to declare.

Authors' contribution: M.F. participated in methodology, patients' recruitment, genetic analysis, investigation, formal analysis, data curation, writing-original draft, and writing-review and editing. G.U. and T.R.N. recruited and investigated patients and revised the manuscript. C.F.Z and S.R.V. conceived the genetic analysis methodology, collected data and revised the manuscript. P.C.M. participated in project administration, funding acquisition, methodology, patients' recruitment, and writing-review. All authors approved the final version of the manuscript.

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