

# Association of proton pump inhibitor use with endothelial function and metabolites of the nitric oxide pathway: A cross-sectional study

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## Abstract

**Study Objective:** Long-term intake of proton pump inhibitors (PPIs) might increase the risk of cardiovascular events. One suggested mechanism is that PPIs inhibit the enzyme dimethylarginine dimethylaminohydrolase (DDAH) and thereby block the degradation of endothelial asymmetrical dimethylarginine (ADMA). Excess ADMA in turn leads to impaired endothelial nitric oxide (NO) generation. So far, this mechanism has only been established in human cell cultures. Previous studies that examined this pathway in human populations measured circulating ADMA and found no association with PPI use and excess plasma ADMA. But in a recent study, plasma ADMA was not correlated with intracellular ADMA. We therefore focused on changes in plasma citrulline as an indicator for potential DDAH inhibition.

**Design:** We analyzed the association between regular daily PPI intake and flow-mediated dilation (FMD) of the brachial artery as well as plasma concentrations of

Nolde, Bahls, Meisinger and Baumeister contributed equally.

[Correction added on February 17 2022, after first online publication: The name of co-author Stefan B. Felix has been corrected to Stephan B. Felix.]

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citrulline, arginine, ADMA, and symmetric dimethylarginine using inverse probability weighting to adjust for confounding and censoring.

**Data Source:** Data of 1298 participants from two independent cohorts of the population-based Study of Health in Pomerania were used.

**Patients:** Participants of the population-based Study of Health in Pomerania are a stratified random sample of the study region.

**Intervention:** Regular daily intake of PPIs.

**Measurements:** FMD of the brachial artery and plasma concentrations of citrulline, arginine, ADMA, and symmetric dimethylarginine.

**Main Results:** Eighty-seven participants (57.5% female) were regular daily users of PPIs. In the fully adjusted models, associations were identified for FMD and plasma citrulline concentrations. PPI users revealed a 0.99% (95% CI: -1.96 to -0.02) lower FMD and 3.03  $\mu\text{mol/L}$  (95% CI: -4.96 to -1.10) lower plasma citrulline levels as compared to non-users.

**Conclusion:** Our data provide evidence that long-term intake of PPIs might inhibit human DDAH activity, resulting in impaired endothelial NO production and reduced vascular function. In the long run, this might explain an increased risk for cardiovascular diseases associated with long-term PPI use.

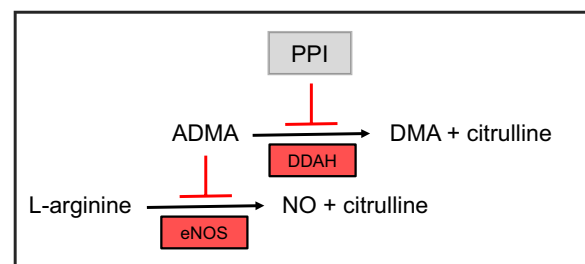
#### KEYWORDS

citrulline, endothelium, flow-mediated dilation, nitric oxide, proton pump inhibitor

## 1 | INTRODUCTION

Proton pump inhibitors (PPIs) are widely used to treat disorders characterized by excessive gastric acid production. For more than 10 years, PPIs have also been sold over the counter and are often consumed without medical supervision. The safety of long-term intake of PPIs has received considerable scientific attention in recent years, as large and well-controlled cohort studies linked PPI use to an increased risk of myocardial infarction and ischemic stroke.<sup>1,2</sup> The elevated risk of myocardial infarction was associated with PPI use but not with the use of histamine H<sub>2</sub> receptor antagonists.<sup>3</sup> However, in the absence of clear evidence for causality and without a mechanism that links PPI use with higher cardiovascular risk, there has been no indication for regulatory authorities to restrict their availability.<sup>4</sup> One approach to improve the evidence is through testing of plausible biological pathways. Several cohort studies indicated that an increased risk of myocardial infarction and ischemic stroke in PPI users was independent of aspirin or clopidogrel intake,<sup>3,5,6</sup> which suggests an underlying mechanism that does not directly involve either platelet aggregation or change in drug absorption due to a rise in gastric pH.<sup>3,5</sup> As a result, potential biochemical mechanisms were put forward that could explain the effect of PPI intake on the cardiovascular system.

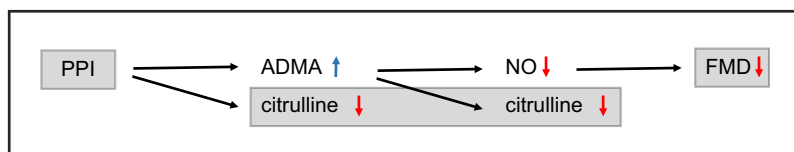
In particular, results from biochemical, cellular, ex vivo and in vivo mouse studies have led to the hypothesis that PPIs may raise intracellular levels of asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide (NO) synthesis, which is accompanied



**FIGURE 1** The role of proton pump inhibitors in endothelial NO synthesis

by lower NO formation, depressed endothelium-mediated vasorelaxation, and increased circulating ADMA.<sup>7</sup> The proposed underlying mechanism is a PPI-dependent direct inhibition of the activity of the major ADMA-degrading enzyme type-1 dimethylarginine dimethylaminohydrolase (DDAH-1),<sup>7</sup> and thereby blocking of the degradation of endothelial ADMA. Excess ADMA in turn leads to impaired endothelial NO generation.

So far, all efforts to reproduce these findings in human studies were without success.<sup>8-10</sup> One possible reason for this is that the plasma concentration of ADMA does not reflect the intracellular endothelial ADMA concentration very well.<sup>11</sup> ADMA might therefore not be the best choice to measure the effect of PPIs on endothelial NO synthesis via DDAH-1 inhibition. A look at the biochemical reactions involved in endothelial NO synthesis (Figure 1) shows that a potential effect of PPIs via DDAH-1 inhibition should most notably



**FIGURE 2** The effect of PPI intake on plasma citrulline and flow-mediated dilation (FMD)

result in a decrease of intracellular L-citrulline,<sup>12,13</sup> as it is involved in both suppressed reactions (Figure 2).

In the current study, we used data from two independent samples of the population-based Study of Health in Pomerania (SHIP) to assess the association between regular daily PPI intake and metabolites of the NO pathway, in particular citrulline, arginine, ADMA, and symmetric dimethylarginine (SDMA); in addition, we analyzed the association between regular daily PPI intake and flow-mediated vasodilation (FMD) of the brachial artery as a measure of endothelial function.

## 2 | METHODS

### 2.1 | Study design and population

SHIP consists of two independent samples, SHIP-0 and SHIP-TREND-0, of adults from a north-eastern German region.<sup>14</sup> For the present analyses, we used data from SHIP-Trend-0 (age range of participants at the examination: 20–79 years) and SHIP-2 (30–90 years), the third examination cycle of SHIP-0, because these studies included measurements of endothelial function (FMD) as well as metabolites of the NO pathway (i.e., citrulline, ADMA, arginine). Data collection took place between 2008 and 2012. Users of clopidogrel and participants who had suffered a previous myocardial infarction or stroke were excluded ( $n = 52$ ) to avoid bias introduced by a potential interaction of clopidogrel with the PPI omeprazole or reverse causation. Due to rare use of PPIs in younger adults, the analysis was restricted to study participants aged  $\geq 38$  years. The final sample consisted of 1298 subjects (Figure S1). The study complies with the Declaration of Helsinki and was approved by the Ethics Committee of the University of Greifswald. Written informed consent was provided by all participants.

### 2.2 | Measures

#### 2.2.1 | Endothelial function

Measurements of vascular function were performed by standardized ultrasound examinations as described in detail elsewhere.<sup>15</sup> In brief, FMD of the brachial artery was assessed. Relative changes are expressed as a percentage of absolute FMD to baseline diameter. FMD values below the 20th percentile (i.e., FMD = 2.29%) were considered decreased.<sup>15</sup> Changes are described in relation to a minimally clinically important difference (MCID) of 1% for FMD, based upon a meta-analysis indicating that 1% increase in brachial FMD decreases the risk of cardiovascular events by 13%.<sup>16</sup>

#### 2.2.2 | Metabolites involved in NO synthesis

Serum concentrations of citrulline, arginine, ADMA, and SDMA were measured using the AbsoluteIDQ p180 Kit (BIOCRATES; Life Sciences AG) at the Institute of Clinical Chemistry and Laboratory Medicine of the University Medicine Greifswald. 10  $\mu$ L aliquots of each plasma sample were processed as recommended by the manufacturer. The kit has proven superior reproducibility in an international ring trial.<sup>17</sup> Samples were excluded from the analysis, if they deviated by more than three standard deviations from the mean. Concentrations of analyzed metabolites are reported in  $\mu$ mol/L.

#### 2.2.3 | PPI intake

Participants were asked to bring to the interview all medications taken in the 7 days preceding the examination. The software IDOM was used to scan the unique pharmaceutical central numbers (Pharmazentralnummer [PZN]). The treatment group consists of participants who reported regular daily PPI intake (Anatomical Therapeutic Chemical [ATC] code A02BC).

#### 2.2.4 | Confounders

We controlled for several confounders, assuming that direct causes of the exposure or outcome, excluding possible instrumental variables, would identify a sufficient set of confounding variables.<sup>18</sup> Accordingly, we adjusted for age, sex, smoking status, alcohol consumption, body mass index (BMI), low-density lipoprotein (LDL), systolic blood pressure, glycated hemoglobin (HbA1c), the estimated glomerular filtration rate (eGFR), high-sensitivity C-reactive protein (hsCRP), antiplatelet therapy (ATC B01AC), intake of statins (ATC C10AA), aspirin,<sup>19</sup> and use of nonsteroidal anti-inflammatory drugs (NSAIDs) (ATC M01A). Use of NSAIDs is a major risk factor for gastroduodenal ulceration and bleeding, and therefore for PPI intake. Studies on the effect of NSAIDs on endothelial function are ambiguous; some identified NSAIDs as risk factors.<sup>20</sup> Moreover, several studies reported associations between GFR and endothelial function, although the causal relation is not clear yet.<sup>21</sup>

Information on age, sex, smoking status (never, former, or current), alcohol consumption habits, medical history (including previous myocardial infarction and stroke), and drug intake were obtained by computer-assisted personal interviews. BMI, defined as body weight divided by height squared [ $\text{kg}/\text{m}^2$ ], and blood pressure were measured at the physical examination.<sup>14</sup> Non-fasting blood samples were taken to determine serum levels of

**TABLE 1** (a) Characteristics of regular daily users of PPIs in the population before and after inverse probability of treatment weighting. (b) Outcome variables before and after inverse probability of treatment weighting

	Unweighted population			Weighted population		
	No PPI	PPI	SMD	No PPI	PPI	SMD
(a)						
<i>n</i>	1211	87		1210	86	
Age [years]	57.05 (11.65)	62.78 (11.21)	0.501	57.43 (11.70)	58.32 (11.15)	0.078
Female (%)	51.1	57.5	0.129	51.5	47.5	0.079
Smoking (%)						
Never	42.0	42.5	0.137	42.0	36.9	0.105
Former	42.0	46.0		42.3	45.3	
Current	16.0	11.5		15.8	17.8	
Alcohol [g/day]	10.17 (13.78)	10.07 (11.84)	0.008	10.07 (13.68)	11.44 (12.68)	0.104
Body mass index [kg/m <sup>2</sup> ]	27.84 (4.27)	29.07 (3.84)	0.302	27.92 (4.31)	28.11 (3.43)	0.048
Low-density lipoprotein [mmol/L]	3.44 (0.91)	3.60 (0.89)	0.182	3.45 (0.92)	3.47 (0.79)	0.024
Systolic blood pressure [mm Hg]	131.27 (18.47)	130.64 (16.56)	0.036	131.21 (18.40)	131.62 (17.11)	0.023
Hemoglobin A1C [%]	5.36 (0.66)	5.55 (0.65)	0.287	5.37 (0.66)	5.38 (0.52)	0.011
Glomerular filtration rate [ml/min]	87.96 (20.06)	79.26 (18.07)	0.456	87.39 (20.05)	85.46 (15.78)	0.107
High-sensitivity C-reactive protein [mg/L]	1.88 (2.87)	2.21 (2.03)	0.134	1.91 (2.97)	2.20 (2.12)	0.116
Antiplatelet drugs (%)	10.2	27.6	0.454	11.4	10.6	0.025
Statins (%)	12.1	26.4	0.368	13.1	12.3	0.023
Aspirin (%)	12.2	28.7	0.418	13.3	12.6	0.021
Nonsteroidal anti-inflammatory drugs (%)	8.8	11.5	0.088	9.0	7.9	0.040
Enrollment in SHIP-Trend-0 (%)	34.1	19.5	0.334	33.2	35.4	0.046
(b)						
<i>n</i>	1211	87		1210	86	
FMD in [%]	5.65 (3.79)	4.80 (3.36)	0.237	5.62 (3.78)	4.63 (3.31)	0.279
Citrulline [μmol/L]	31.57 (8.81)	29.63 (9.44)	0.213	31.62 (8.84)	28.59 (7.93)	0.361
Arginine [μmol/L]	75.31 (19.28)	72.46 (17.09)	0.156	75.33 (19.27)	72.39 (15.34)	0.169
ADMA [μmol/L]	0.51 (0.16)	0.50 (0.10)	0.074	0.51 (0.16)	0.49 (0.12)	0.089
SDMA [μmol/L]	0.53 (0.25)	0.56 (0.24)	0.145	0.53 (0.25)	0.53 (0.26)	0.023

Note: (a) Continuous variables as [mean (standard deviation)]. (b) All variables as [mean (standard deviation)].

Abbreviations: ADMA, asymmetric dimethylarginine; FMD, flow-mediated dilation; PPI, proton pump inhibitors; SDMA, symmetric dimethylarginine; SMD, standardized mean difference.

LDL cholesterol, creatinine, and hsCRP. HbA1c was determined by high-performance liquid chromatography. The eGFR was calculated according to Levey et al.<sup>22</sup>

### 2.3 | Statistical analysis

We used inverse probability of treatment (IPT) weighting to adjust for confounding.<sup>23</sup> Propensity scores were estimated from a confounder-adjusted logistic regression and used to calculate

stabilized weights.<sup>24</sup> For reporting the balance in each individual covariate between treated and reference populations, the standardized difference in prevalence or means was calculated. IPT-weighted linear regression models with robust standard errors were used to associate regular daily PPI intake with FMD, citrulline, arginine, ADMA, and SDMA. We used IPT-weighted quantile regression with bootstrapped standard errors to examine the association between PPI use and decreased FMD. We examined additive effect modification by adding treatment-covariate product terms to the marginal structural model.

In additional analyses, we used inverse probability of censoring (IPC) weighting to account for possible differential dropout from baseline. Stabilized IPC weights were estimated using logistic regression models and combined with IPT weights.<sup>24</sup> These combined IPT/IPC weights were used to adjust the models for the association of PPI intake and FMD, citrulline, arginine, ADMA, and SDMA levels for confounding and censoring. We assessed the robustness of observed associations to unmeasured confounding. Specifically, we calculated the E-value,<sup>25</sup> which indicates the minimum strength of the association that an unmeasured confounder would need to have with the exposure and the outcome on the risk ratio scale to fully account for the observed exposure-outcome association, above and beyond measured confounders. The statistical software R (version 3.6.2, Foundation for Statistical Computing, Vienna, Austria) was used.

### 3 | RESULTS

Of the 1298 analyzed subjects (51.5% female), 87 participants (57.5% female) were regular daily users of PPIs. The predominant PPIs were omeprazole ( $n = 48$ , ATC code A02BC01) and pantoprazole ( $n = 31$ , ATC code A02BC02). Confounders in PPI users and non-users, respectively, are presented in Table 1a. Before IPT-weighting, participants who reported PPI intake were older, had higher BMI, lower eGFR, and were more likely to use other medications. After IPT-weighting, the PPI and non-PPI populations were well balanced on the confounders. Raw and adjusted outcome variables are presented in Table 1b.

#### 3.1 | The association of PPI intake with FMD, citrulline, arginine, ADMA, and SDMA

In PPI users, FMD and citrulline were reduced. Adjusted for all confounders PPI users had a 0.99% (95% confidence interval [CI]:

**TABLE 2** Fully adjusted estimates for the association between the regular daily intake of PPIs and FMD, as well as plasma concentrations of citrulline, arginine, ADMA, and SDMA

Outcome	Regression coefficient	95% CI	<i>p</i>
FMD in [%]	-0.99	-1.96; -0.02	0.045
Citrulline [ $\mu\text{mol/L}$ ]	-3.03	-4.96; -1.10	0.002
Arginine [ $\mu\text{mol/L}$ ]	-2.94	-6.86; 0.98	0.142
ADMA [ $\mu\text{mol/L}$ ]	-0.01	-0.05; 0.02	0.509
SDMA [ $\mu\text{mol/L}$ ]	0.01	-0.06; 0.07	0.865

Note: Method: inverse probability of treatment weighted linear regression with stabilized weights using all the covariates of Table 1. Abbreviations: ADMA, asymmetric dimethylarginine; FMD, flow-mediated dilation; PPIs, proton pump inhibitors (Anatomical Therapeutic Chemical (ATC) code A02BC); SDMA, symmetric dimethylarginine.

-1.96 to -0.02) lower FMD, which matches our MCID of 1%, and a 3.03  $\mu\text{mol/L}$  (95% CI: -4.96 to -1.10) lower plasma citrulline level compared to non-users (Table 2). This reduction is equivalent to 0.26 standard deviations for FMD and 0.34 standard deviations for citrulline. PPI intake was not associated with changes in plasma concentrations of arginine, ADMA, and SDMA. An additional analysis using propensity score matching gave similar estimates (FMD: -0.94%; Citrulline: -2.56  $\mu\text{mol/L}$ ) and slightly wider confidence intervals (FMD: -2.01 to 0.13%; Citrulline: -5.36 to 0.24  $\mu\text{mol/L}$ ). The quantile regression model indicated that the relation of PPI use with decreased FMD was -1.18% (95% CI: -2.18 to -0.17). The effect of PPI intake on FMD and citrulline was not modified by systolic blood pressure or smoking status.

Analyses that additionally adjusted for censoring using inverse probability weighting were in line with our primary results (Table S1). In the IPT-weighted linear model for PPI intake and FMD in order to explain the regression coefficient of -0.99%, an unmeasured confounder would have to be associated with PPI intake by a risk ratio of 1.89-fold, above and beyond the measured confounders. For an unmeasured confounder to explain the association between PPI use and citrulline, the unobserved confounder would need to be related to PPI use with a risk ratio of 1.91.

### 4 | DISCUSSION

Our study found that regular daily PPI use was associated with lower vascular endothelial function and lower plasma citrulline concentrations. FMD in PPI users was on average 0.99% lower and plasma citrulline levels were reduced by 3.03  $\mu\text{mol/L}$  compared to non-users. In relation to study population means of 5.59% for FMD and 31.46  $\mu\text{mol/L}$  for citrulline, these differences are quite substantial. Quantile regression for the 0.2 quantile showed that the effect on vascular function is even stronger in individuals with decreased FMD (FMD below 2.29%), where FMD was 1.18% lower among PPI users compared to non-users. In comparison, a meta-analysis found that the pooled relative risk of cardiovascular events per 1% increase in brachial artery FMD, adjusted for confounding factors, was 0.87.<sup>16</sup> The E-value of 1.9 for both FMD and citrulline implies that in order to explain away the observed associations an unmeasured confounder would have to be associated with PPI intake by a risk ratio of 1.9-fold, above and beyond the measured confounders. The strongest confounder in our logistic model for estimating the propensity for PPI intake was antiplatelet therapy, which was associated with PPI use with an odds ratio of 0.87 (approximately equivalent to an inverted risk ratio of 1.15, [i.e., 0.87<sup>-1</sup>]). We found no effect modification on FMD or citrulline by systolic blood pressure or smoking status. In line with previous studies, there was no association between PPI intake and plasma levels of ADMA.<sup>8-10</sup> We are not aware of any studies that assessed the effect of PPI intake on plasma SDMA or arginine, and our data showed no relationship between these variables.

In biochemical, cellular, ex vivo, and mouse studies, PPIs were found to directly inhibit the enzyme DDAH and thereby elevate the intracellular concentration of ADMA.<sup>7</sup> High ADMA levels are posing an increased cardiovascular risk by inhibiting the endothelial nitric oxide synthase (eNOS) resulting in reduced production of NO and endothelial dysfunction.<sup>26–28</sup> Unexpectedly, studies that sought to replicate these findings in humans by hypothesizing higher plasma ADMA levels and reduced endothelial function in PPI users have been unsuccessful.<sup>9,10</sup> Subsequent research therefore focused on the proposition of alternative mechanisms, such as accelerated endothelial aging.<sup>29,30</sup> One possible explanation is that circulating ADMA levels are not strongly correlated with intracellular ADMA, which is supposed to be affected by PPI intake.<sup>11</sup> NO itself is very reactive and complex to measure directly. Perhaps citrulline could be a suitable marker for the whole system of involved reactions leading to reduced endothelial NO synthesis as it is a product of the inhibited degradation of ADMA as well as of the inhibited production of NO.<sup>7,13</sup> Citrulline is involved in the metabolism of ADMA by the enzyme DDAH, leading to citrulline and DMA. In addition, it is a product of the NO synthesis, where arginine is degraded by eNOS into NO and citrulline. Endothelial citrulline levels are therefore twofold decreased by DDAH inhibition and may show the biggest response to PPI intake. Vascular function on the other hand might predominantly be affected by long-term intake of PPIs, exceeding the 4 weeks of treatment, that were covered in a previous study.<sup>10</sup> With that in mind, we used plasma citrulline as an indicator for endothelial DDAH inhibition and assessed the resulting effect of regular daily PPI intake on flow-mediated brachial artery endothelial function.

In this context, our results support the notion that PPI intake might increase cardiovascular risk by lowering NO availability in endothelial cells and thus are in line with previous experimental research.<sup>7</sup> This is the first stringent evidence for PPI caused DDAH inhibition resulting in reduced endothelial function in humans.

Our study has several limitations. First, due to the cross-sectional design, regular daily PPI use and markers of endothelial function were measured at the same time. Lacking respective measurements at treatment initiation, we cannot assure that PPI exposure precedes the purported effects in time or exclude the possibility that lowering of FMD and citrulline levels had already occurred. Second, we had no information about start and duration of PPI intake and used self-reported regular daily intake to identify long-term users of PPI in our study. This approach left us with only 87 regular daily users of PPI (out of 1298) and might have induced selection bias in the form of prevalent user bias.<sup>31</sup> In addition, modeling prevalent PPI use in the analysis implies that confounders are measured after treatment initiation. If confounders are affected by prior treatment, this could bias the direct effect estimates of PPI intake.<sup>18</sup> We excluded users of clopidogrel, and individuals with prior myocardial infarction or stroke, to avoid bias due to a potential interaction of clopidogrel with the PPI omeprazole or reverse causation. Although our sensitivity analysis indicates adjustment for a sufficient set of confounders, unmeasured confounding might still have been reduced by adjusting

for further risk factors (e.g. physical exercise, depression, anxiety, fatigue) or choosing histamine H2 receptor antagonists as an active comparator. Sadly, this was not possible, as the study population included only 1 regular daily user of H2 receptor antagonists.

In summary, we provide evidence that long-term intake of PPIs might inhibit human DDAH activity and thereby impair endothelial NO production and vascular endothelial function. Chronic disruption of eNOS may explain an increased risk for cardiovascular events among long-term users of PPIs, as found in several studies.<sup>1,6</sup> As PPIs play an important role in the treatment of gastric acid-related disorders, more insight into the consequences of long-term intake is desired. Studies that relate new PPI intake with the variation of endothelial function and plasma citrulline levels measured over time could deliver further evidence for PPI-induced DDAH inhibition in humans and explain the potential for increased cardiovascular risk in long-term PPI users.

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#### CONFLICT OF INTEREST

The authors have no conflicts of interest to disclose.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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