Citrulline and long-term mortality in patients with cardiovascular disease

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None declared

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Abstract

Background. Cardiovascular disease (CVD) is associated with intestinal barrier dysfunction and increased intestinal permeability. Increased intestinal permeability to gut microbial metabolites may accelerate the progression of CVD. Plasma citrulline levels are a marker of functional enterocyte mass, and reduced citrulline levels indicate intestinal epithelial damage. Citrulline was reported as a useful prognostic marker in critically ill patients. However, data are lacking on the association of citrulline with long-term mortality in patients with CVD and with the levels of trimethylamine N-oxide (TMAO), a microbiota-derived metabolite which has been implicated in the pathogenesis of CVD.

Objectives. To assess the effect of citrulline levels, a marker of intestinal barrier disruption, on long-term mortality in patients with CVD. Moreover, the relationship between the concentrations of 2 biomarkers – citrulline and TMAO – was assessed.

Materials and methods. Serum citrulline levels were retrospectively assessed in 1036 consecutive patients with CVD (median age: 62 years; 61% men) hospitalized between 2013 and 2015. Associations of citrulline levels with 5-year mortality rates as well as anthropometric and biochemical parameters were evaluated for the entire study group and in subgroups of patients with acute coronary syndrome (ACS), chronic coronary syndrome, chronic heart failure (chronic HF), and atrial fibrillation (AF). Correlations between serum citrulline and TMAO levels were assessed.

Results. The median citrulline level in the study population was 22.5 μM (interquartile range (IQR): 17.8–27.9). Citrulline levels were not associated with 5-year mortality in patients with CVD (hazard ratio (HR) = 0.99; 95% confidence interval (95% CI): 0.97–1.00; p = 0.49). Median citrulline levels differed significantly between deceased patients and survivors at 5 years in patients with ACS (p = 0.025). There were no significant correlations between citrulline and TMAO levels (Kendall's tau = 0.027).

Conclusions. Decreasing citrulline levels do not predict long-term mortality of hospitalized patients with CVD. Moreover, they are not associated with the serum levels of TMAO in these patients.

Key words: cardiovascular diseases, intestinal barrier, citrulline, gut permeability, TMAO

Background

Cardiovascular disease (CVD) has been reported to be associated with intestinal barrier dysfunction and increased intestinal permeability.[1] Initial studies focused on altered intestinal function in patients with chronic heart failure (chronic HF).[2–4] However, a link between intestinal barrier dysfunction and coronary artery disease,[5,6] ST-segment elevation myocardial infarction[7,8] and arterial hypertension[9,10] has also been reported. A vicious cycle has been described wherein CVD impairs the intestinal barrier, making it more permeable to toxic substances that favor the progression of cardiovascular abnormalities.[1]

The assessment of the intestinal barrier competency is a complex task which can be achieved using a number of approaches.[11] One approach is to use biomarkers such as bacterial lipopolysaccharide (LPS),[11] zonulin,[12] claudins,[13] and intestinal fatty acid binding protein.[14] There are also tools for evaluating the function of intestinal barrier samples such as the Ussing chamber.[15] Depending on the method used, various structural and functional parameters can be measured. One available biomarker of intestinal barrier function is serum citrulline level, which allows for an indirect assessment of absorptive enterocyte mass.[1,16–18] Citrulline is an amino acid synthesized mainly by enterocytes in the proximal small bowel, in the middle and upper parts of intestinal villi.[17] Citrulline measurements have been reported to be a useful marker of acute mesenteric ischemia.[19] Moreover, their use as a marker of epithelial lining damage in a human model of small intestinal ischemia and reperfusion has been described.[20] Reduced citrulline levels also serve as a biomarker of intestinal barrier failure and are an independent prognostic factor in critically ill patients.[21–24]

A recent meta-analysis of 26 randomized controlled studies found that long-term citrulline supplementation significantly improves vascular endothelial function and reduces arterial stiffness.[25] As the authors point out, these effects are associated with a reduced risk of cardiovascular events. Other beneficial effects of citrulline supplementation include improved blood pressure, glucose and lipid profile, and the bioavailability of arginine and nitric oxide.[26]

So far, no studies have assessed the use of citrulline levels as a predictor of long-term mortality in patients with CVD. Moreover, data are lacking on the association between levels of citrulline and the microbiota-derived metabolite trimethylamine N-oxide (TMAO).[27,28] It was postulated that intestinal permeability can significantly affect the absorption of TMAO and its precursors.[29] The current study is a continuation of our previous research on the same population of patients (unpublished data). The previous study did not reveal a significant association between TMAO levels and long-term mortality in CVD patients. The assessment of intestinal permeability may shine a new light on our previous findings and guide the direction of future research.

Objectives

This study aimed to assess the effects of citrulline levels, a marker of enterocyte functional mass, on the long-term mortality of CVD patients. Moreover, the relationship between 2 biomarkers – citrulline and TMAO levels – was evaluated.

Materials and methods

Patients

The study included 1036 consecutive patients hospitalized between March 2013 and November 2015 in the Department of Cardiology at Wroclaw Medical University in Wrocław, Poland. All patients provided written informed consent to participate in the study and submitted blood samples for laboratory testing.

The exclusion criterion was the lack of patient's informed consent. Patients who did not want to consent or were unable to consent due to their clinical condition (i.e., unconscious or intubated) were excluded from the study. We routinely included patients on days 1 or 2 of the hospital stay in order to enroll patients with stable conditions who were willing to participate in the study.

Specimen characteristics

Blood samples were stored at a temperature of –80°C at the BioBank of the Łukasiewicz Research Network – PORT Polish Center for Technology Development in Wrocław, Poland.

Assay methods

Liquid chromatography with tandem mass spectrometry was used to determine serum L-citrulline and TMAO levels. Briefly, samples were thawed on ice and extracted by adding a mix of d4-citrulline and d9-TMAO (Cambridge Isotope Laboratories, Tewksbury, USA) in acetonitrile (final concentration 10 μM) at a ratio of 1:3 (v/v). The chromatographic separation was performed using a Luna Silica analytical column (3 μm, 100 Å, 150 × 2 mm; Phenomenex, Torrance, USA) and the UltiMate™ 3000 UPLC system (Dionex, Sunnyvale, USA). An isocratic elution of the mobile phase consisting of 0.1% formic acid in acetonitrile and water at a ratio of 60:40 (v/v) was applied at a flow rate of 0.3 mL/min (total run time: 5 min). A multiple reaction monitoring mode was selected for mass spectrometric detection using ESI-Q-TOF (Bruker Daltonics, Bremen, Germany) in a positive ion mode. A calibration curve was constructed at a range of 0.5–100 μM for L-citrulline (Sigma-Aldrich, St. Louis, USA) and 0.125–25 μM for TMAO.

This method has been validated according to the Food and Drug Administration (FDA) guidelines.[30] The linearity

was determined by using correlation coefficients (R^2) which were 0.9997 for both citrulline and TMAO levels. The sensitivity of the laboratory analysis was assessed based on the limit of detection and limit of quantification. These were 0.221 μM and 0.662 μM, respectively, for citrulline and 0.236 μM and 0.708 μM, respectively, for TMAO. The precision and accuracy of the measurements were assessed with the use of quality control samples and standard solutions of various concentrations. The calculated inter- and intra-assay coefficients of variation did not exceed 8% for any of the tested levels, and the accuracy was in the range of 95–105%. This is in line with the limits proposed for the validation of assay methods for biological samples.

All samples were measured in 2 biological and 2 technical replicates. The peak area ratio (analyte/internal standard) was used for the calculation of mean L-citrulline and TMAO levels in samples. Data on current diagnosis, comorbidities, anthropometric parameters, laboratory test results, and medication use were obtained from the medical records of the index hospitalization.

Study design

This is a retrospective observational study.

At the time of hospitalization, patients donated blood samples for future research. The protocol of sampling and biobank creation was approved by the local ethics committee of Wroclaw Medical University on February 9, 2011 (approval No. 73/2012). All patients consented to the storage and processing of samples for future studies.

The concentrations of the selected biomarkers were assessed retrospectively in collected samples. Mortality was analyzed using the registry of the Polish National Health Fund (as of February 19, 2020).

Statistical analyses were performed to assess the correlation of biomarker concentrations with mortality and the available clinical data obtained during the hospitalization of each patient.

The study protocol was approved by the local ethics committee of Wroclaw Medical University on February 28, 2019 (approval No. 163/2019). The study was conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent to participate in the study.

Statistical analyses

The statistical analyses were performed on the whole population. Subgroups of patients were classified according to the main clinical diagnosis of CVD: 1) patients with acute coronary syndrome (ACS) during the index hospitalization; 2) patients with chronic coronary syndrome; 3) patients with chronic HF without ACS; and 4) patients with atrial fibrillation (AF).

Additionally, patients were classified according to the occurrence of death during the follow-up period, as shown in Table 1 for the whole study group, and within subpopulations, as shown in Table 2. Depending on data distribution, quantitative data were presented as means with standard deviations (SDs) and medians with interquartile ranges (IQRs). The rates of CVD and medication use were presented as percentages of patients. The normality of data distribution was assessed using the Kolmogorov–Smirnov and Lilliefors tests. The Kendall's tau test was used to assess the correlation between variables. The mean values of 2 independent variables were compared using the nonparametric Mann–Whitney test, while the Kruskal–Wallis analysis of variance (ANOVA) was used to compare more than 2 independent variables. The effect of continuous variables on mortality in subgroups was assessed using the Cox proportional hazards model. The proportional hazards assumptions were tested with proportional tests and presented graphically using the plots of scaled Schoenfeld residuals. The goodness-of-fit was tested by calculating the coefficient of determination (R^2). The Cox proportional hazards model with interactions was used to assess the independence of variables on mortality prediction. Survival curves for individual variables were generated using the Kaplan–Meier estimator. A value of $p < 0.05$ was considered statistically significant. All analyses were performed using STATISTICA v. 13 software (TIBCO Software Inc., Palo Alto, USA; https://www.tibco.com/).

Results

The characteristics of the study population are presented in Table 1. The study included a total of 1036 patients, including 177 (11.3%) patients with ACS, 441 (42.6%) patients with chronic coronary syndrome, 292 (28.2%) patients with chronic HF, and 277 (26.7%) patients with AF. The mean follow-up for the study group was 58.4 months. The 5-year mortality rate for the whole population was 16.5% (171 deaths). Most of the clinical and biochemical parameters differed between the deceased patients and the survivors (Table 1).

There were no significant differences in citrulline levels between subgroups divided according to survival (Table 1). Moreover, the univariate Cox proportional hazards analysis revealed no association between citrulline levels and death at 5 years (hazard ratio (HR) = 0.99; 95% confidence interval (95% CI): 0.97–1.00; p = 0.49). Therefore, a multivariate analysis was not performed.

For a more detailed analysis, citrulline levels were divided into quartiles. However, no differences were noted between the quartiles in terms of their effect on survival at 5 years (χ^2 test = 3.53; p = 0.31) (Fig. 1).

To obtain a more detailed insight into the effect of citrulline levels on mortality in patients with CVD, we performed a subgroup analysis of citrulline levels. The differences in citrulline levels in subgroups divided according to survival at 5 years are presented in Table 2. Significantly

Table 1. Study population characteristics

Parameter		Study population (n = 1036)	Death at 5 years		p-value*
			yes (n = 171)	no (n = 865)	
Age [years], mean (SD)		62.0 (14.1)	68.9 (11.1)	60.7 (14.3)	<0.0001
Male sex [%]		61.1	73.0	58.8	<0.001
TMAO [µmol/L]		4.06 (2.79–6.01)	5.65 (3.48–8.94)	3.86 (2.70–5.62)	<0.0001
Citrulline [µmol/L]		22.5 (17.8–27.9)	21.5 (16.5–27.6)	22.8 (18.0–28.0)	0.18
Acute coronary syndrome [%]		17.0	32.7	19.4	<0.001
Chronic coronary syndrome [%]		42.5	57.1	40.0	<0.0001
Chronic HF [%]		28.1	54.7	23.4	<0.0001
Atrial fibrillation [%]		26.7	43.2	23.4	<0.0001
Other types of arrhythmia [%]		16.4	14.7	85.2	0.48
Conduction disorders [%]		44.0	13.1	86.8	0.01
PCI during hospitalization [%]		21.0	31.1	23.3	0.051
Previous PCI [%]		14.4	25.7	16.9	0.017
Previous CABG [%]		7.6	12.2	6.7	0.012
Diabetes [%]		24.7	35.2	22.6	<0.001
Chronic kidney disease [%]		14.58	28.5	71.5	0.002
Dialysis patients [%]		1.45	27.67	73.33	0.001
Hypertension [%]		71.7	81.8	69.7	0.001
Smoking (current) [%]		23.2	30.0	21.9	0.026
NYHA (% of patients with chronic HF)	I	7.0	8.7	6.3	0.16
	II	55.5	48.3	58.8	
	III	34.0	37.7	32.3	
	IV	3.3	5.3	2.6	
CCS (% of patients with chronic coronary syndrome)	I	10.2	6.0	11.3	0.65
	II	54.3	57.5	53.5	
	III	32.2	33.3	32.0	
	IV	3.1	3.0	3.1	
LVEF [%]		60 (50–65)	50 (37–65)	65 (55–66)	<0.0001
eGFR [mL/min/1.73 m^2]		68 (55–80)	58 (44–72)	69 (58–81)	<0.0001
hsCRP [mg/L]		3.47 (1.33–13.3)	7.79 (2.64–30.8)	3.11 (1.24–10.1)	<0.0001
HbA_{1c} [%]		5.70 (5.40–6.20)	5.90 (5.50–6.65)	5.70 (5.40–6.10)	0.018
TC [mg/dL]		182 (149–216)	171 (135–207)	184 (151–218)	0.003
LDL-C [mg/dL]		104 (80–136)	97 (33–249)	106 (83–136)	0.094
HDL-C [mg/dL]		45 (37–55)	42 (34–51)	46 (38–55)	<0.001
Triglycerides [mg/dL]		124 (95–169)	115 (89–155)	127 (96–172)	0.018
ASA [%]		54.8	64.0	52.9	0.009
Clopidogrel [%]		29.4	28.1	35.1	0.075
OAC [%]		28.9	43.4	26.0	<0.0001
ACEIs [%]		70.2	76.1	69.0	0.066
ARBs [%]		7.19	3.01	8.02	0.022
β-blockers [%]		81.2	92.2	79.0	<0.0001
Statins [%]		79.1	89.2	77.0	<0.001
Loop diuretics [%]		19.1	43.4	14.3	<0.0001

Data are presented as median and interquartile range (IQR) unless indicated otherwise. ACEI – angiotensin-converting enzyme inhibitor; ARB – angiotensin receptor blocker; ASA – acetylsalicylic acid; CABG – coronary artery bypass grafting; CCS – Canadian Cardiovascular Society grading of angina pectoris; eGFR – estimated glomerular filtration rate; HbA_{1c} – glycated hemoglobin A_{1c}; HDL-C – high-density lipoprotein cholesterol; HF – heart failure; hsCRP – high-sensitivity C-reactive protein; LDL-C – low-density lipoprotein cholesterol; LVEF – left ventricular ejection fraction; NYHA – New York Heart Association Functional Classification; OAC – oral anticoagulation; PCI – percutaneous coronary intervention; SD – standard deviation; TC – total cholesterol; TMAO – trimethylamine N-oxide.

Table 2. Citrulline levels in subgroups of deceased patients compared to survivors at 5 years

Subgroup	Whole subgroup [μmol/L]	Death at 5 years		p-value
Acute coronary syndrome (n = 177)	23.3 (18.3–29.1)	yes (n = 37)	no (n = 140)	0.025
		20.9 (15.2–25.8)	24.0 (18.5–30.1)	
Chronic coronary syndrome (n = 441)	22.2 (17.5–28.1)	yes (n = 96)	no (n = 345)	0.12
		21.0 (15.7–21.3)	22.4 (18.0–28.4)	
Chronic HF (n = 292)	22.2 (17.9–28.2)	yes (n = 93)	no (n = 199)	0.50
		21.7 (16.6–28.2)	22.3 (18.3–28.3)	
Atrial fibrillation (n = 277)	22.2 (17.7–28.5)	yes (n = 74)	no (n = 203)	0.89
		22.9 (16.5–30.1)	22.1 (18.1–28.4)	

Data are presented as median and interquartile range (IQR). HF – heart failure.

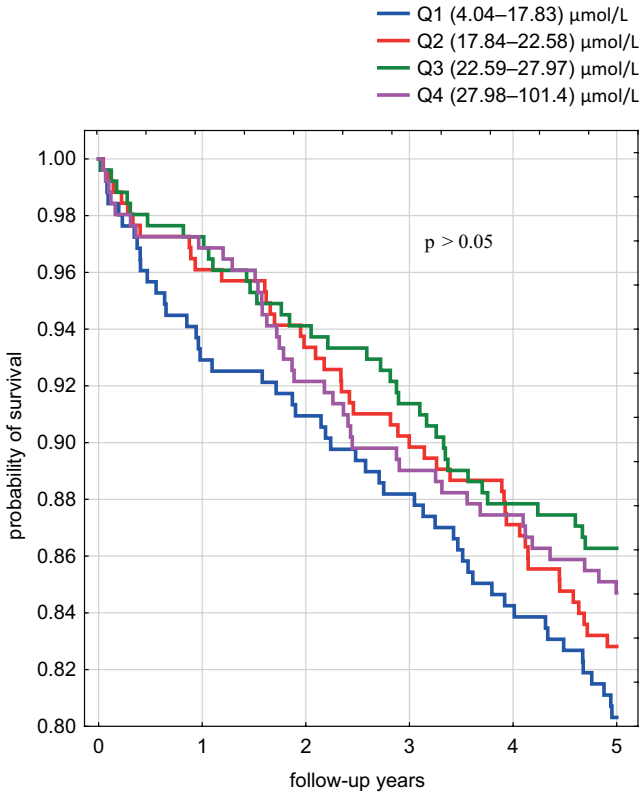

Fig. 1. Kaplan–Meier survival curves in subgroups divided according to quartiles of citrulline levels (Q1, Q2, Q3, and Q4 – quartiles 1, 2, 3, and 4, respectively).

lower citrulline levels were observed in deceased patients compared to survivors at 5 years in patients with ACS. However, the univariate Cox proportional hazards analysis did not reveal any significant associations between citrulline levels and the risk of death at 5 years in any of the subgroups, including patients with ACS. Therefore, multivariate analysis was not performed.

Our analysis revealed no significant correlations between citrulline levels and any of the recorded clinical parameters. Additionally, there were no significant correlations between citrulline levels and multimorbidity, defined as the number of comorbidities present in 1 patient, including ACS, stable coronary syndrome (SCS), chronic HF, AF, arterial hypertension, arrhythmias, and/or conduction disorders. Finally, no significant correlations were noted between TMAO and citrulline levels (Kendall's tau = 0.027).

Discussion

Our study indicates that serum citrulline levels are not correlated with long-term mortality in patients hospitalized due to CVD. While several markers of intestinal barrier dysfunction are available, this study was constructed to assess a single parameter. We decided on citrulline because there is limited research available on the association between citrulline levels and long-term outcomes in CVD patients.

When investigating a single marker of intestinal permeability, the interpretation of the results must account for some inherent limitations. One of the most common markers of an impaired intestinal barrier is LPS.[11] However, LPS assessment in peripheral blood is relatively difficult and often produces false positive results. Moreover, similarly to anti-endotoxin antibodies, it serves only as an indirect marker of increased intestinal permeability.[11] Another known biomarker is intestinal fatty acid-binding protein. However, there are limited data supporting its use in the assessment of chronic intestinal barrier disruption.[31] As for zonulin, previous studies have used commercial enzyme-linked immunosorbent assays (ELISAs) for zonulin measurements. However, these assays do not reflect the actual levels of zonulin but rather the levels of a structurally similar haptoglobin.[12] The reliability of studies on zonulin is further limited by the fact that zonulin is not expressed in mice. Thus, the results obtained actually reflect the levels of unknown proteins rather than zonulin.[12] Finally, claudins are a highly diverse class of 27 proteins present in numerous tissues.[13] They have not only low tissue specificity but also opposing functions, making it difficult to choose a specific protein and interpret the results.[13]

The usefulness of citrulline for the direct functional assessment of enterocyte mass has been confirmed in previous research studies.[16,32] According to the literature, citrulline cutoff values have a sensitivity of 80% and

a specificity of 84% for diagnosing intestinal dysfunction. The most commonly used threshold for low citrulline levels is 20 μmol/L,[16] which is in line with our findings. According to other investigators, a citrulline threshold of 10 μmol/L indicates a significant loss of enterocyte mass.[32] Finally, it has been reported that citrulline levels ranging from 10 μmol/L to 20 μmol/L are a grey zone for interpretation in critically ill patients.[33] Citrulline level of 40 ±10 μmol/L is considered normal.[32] The abnormal citrulline levels in our population may indirectly suggest intestinal epithelial damage. However, several other factors need to be considered when interpreting these results.

Despite high susceptibility to blood supply disorders, high regenerative capacity of the intestinal epithelial lining after ischemia and reperfusion has been reported.[20] So far, citrulline assays have been used mainly for the assessment of critically ill patients.[22–24,34,35] Changes in citrulline levels have been associated with patient prognosis[24] and enteral nutrition.[35] However, these studies were limited by a shorter duration of follow-up compared with our current research.

Considering the currently available literature, the fact that the diagnostic and prognostic values of citrulline are higher in acute states than in chronic ones cannot be excluded. At the same time, reduced citrulline levels are typically observed in chronic diseases associated with intestinal epithelial loss, such as Crohn's disease, celiac disease and short bowel syndrome.[16] It is possible that changes in citrulline levels during the course of chronic CVD result in different dynamics and are induced by different mechanisms. This seems to be supported by the lack of correlation between citrulline levels and multimorbidity in our study. A significant difference in citrulline levels between deceased patients and survivors was noted only in patients with ACS, the only subgroup with an acute cardiovascular condition in our study. Previous studies on patients with myocardial infarctions reported abnormal levels of other markers indicating impaired intestinal barrier, such as LPS, D-lactate, zonulin, and endotoxin.[7,8,36] Moreover, Zhou et al. reported that LPS and D-lactate were associated with a higher risk of mortality at 3 years.[8] In our study, citrulline was not related to mortality risk at 5 years even in patients with ACS. The discrepancy between our study and the study by Zhou et al. may result from differences in the type of biomarkers and frequency of measurements. Zhou et al. assessed the levels of LPS and D-lactate at 2 time points over consecutive days.[8] Our study, on the other hand, involved a single measurement of citrulline levels.

Serial measurements have advantages over a single assessment. Previous studies assessing changes in citrulline levels have revealed correlations between citrulline and the clinical status of patients.[24,35] In an experimental study by Park et al., serial measurements reduced the potential confounding effect of circadian variation and fluctuations in citrulline levels.[37]

Our study did not reveal any correlation between citrulline levels and clinical parameters such as estimated glomerular filtration rate (eGFR) or C-reactive protein (CRP) levels. This is surprising because kidney disorders and enhanced inflammatory processes may induce changes in citrulline levels.[32] Citrulline is converted to arginine in the kidneys. Arginine is then used in nitric oxide synthesis, for example, in response to inflammation.[32] Rare metabolic disorders and partial small bowel resections resulting in short bowel syndrome can affect citrulline levels, but these conditions were excluded from our study population. Only 2 patients had Crohn's disease and there were no cases of celiac disease or short bowel syndrome in the population examined in our study. Therefore, citrulline levels were not affected by these factors in our patients.

In our opinion, this study increases the current knowledge on the absorption of gut microbial metabolites. A previous prospective study by Kitai et al. revealed a significant association between worse prognosis and increased intestinal permeability assessed using the lactulose/rhamnose permeability test.[38] However, the sample size was relatively small (29 patients with HF) and the follow-up was shorter than in our study (median: 56 days). The increased intestinal permeability was not associated with levels of TMAO and intestinal fatty acid binding protein.[38] The lack of correlation between citrulline and TMAO levels may be explained by a yet unknown mechanism of TMAO uptake through the intestinal wall, independent of the number of enterocytes. However, this would be surprising because enterocyte mass reduction should result in the disruption of organic cation transporters mediating TMAO uptake.[39,40]

Limitations

The major limitation of our study is that only a single measurement of citrulline was performed due to the retrospective study design and available biologic material. Optimally, the assessment of several different markers indicating intestinal barrier function performed at different timepoints depending on the duration of short-term and long-term follow-up needs to be conducted. A histopathological examination of an intestinal mucosa biopsy or the functional assessment of gastrointestinal absorption might also provide additional valuable data.

The strength of our study was its large sample size, the use of multiple clinical parameters, and the long-term follow-up. Additionally, the samples were obtained in a daily clinical practice setting and the citrulline levels were assessed using validated and reproducible methods.

Conclusions

Despite the limitations inherent to its retrospective design, our study provides novel insights into intestinal barrier dysfunction and fills the existing gap in the literature.

Decreased citrulline levels do not predict long-term mortality of hospitalized patients with CVD. Moreover, they are not associated with serum TMAO levels in these patients. We believe that these findings will provide a basis for future research into the links between intestinal permeability and the prognosis of CVD patients. The use of citrulline levels in conjunction with morphological and functional assessments of intestinal biopsies in CVD patients would allow for the assessment of potential new correlations. Perhaps serial measurements of citrulline concentrations during hospitalization and shorter follow-up will allow to capture the dynamics of the processes affecting the intestinal barrier and prognosis of patients with CVD.

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