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REVIEW

Gut microbiota-dependent phenylacetylglutamine in cardiovascular disease: current knowledge and new insights

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Abstract **Phenylacetylglutamine (PAGln) is an amino acid derivate that comes from the amino acid phenylalanine. There are increasing studies showing that the level of PAGln is associated with the risk of different cardiovascular diseases. In this review, we discussed the metabolic pathway of PAGln production and the quantitative measurement methods of PAGln. We summarized the epidemiological evidence to show the role of PAGln in diagnostic and prognostic value in several cardiovascular diseases, such as heart failure, coronary heart disease/atherosclerosis, and cardiac arrhythmia. The underlying mechanism of PAGln is now considered to be related to the thrombotic potential of platelets via adrenergic receptors. Besides, other possible mechanisms such as inflammatory response and oxidative stress could also be induced by PAGln. Moreover, since PAGln is produced across different organs including the intestine, liver, and kidney, the cross-talk among multiple organs focused on the function of this uremic toxic metabolite. Finally, the prognostic value of PAGln compared to the classical biomarker was discussed and we also highlighted important gaps in knowledge and areas requiring future investigation of PAGln in cardiovascular diseases.**

Keywords PAGln; cardiovascular disease; gut microbiota; uremic metabolite; biomarker

Introduction

The gut microbiota is described as the "microbial organ" of the human body, which participates in the host's energy metabolism, regulates innate and acquired immunity, and is regarded as the biological barrier of the human intestine [1]. Recently, two "back-to-back" randomized controlled trials found that the occurrence of cardiovascular disease is closely related to changes in the host intestinal microbiota [2], and intestinal microbiota dysbiosis often appears earlier than the clinical manifestations [3], confirming that intestinal microbial imbalance may be the initiation of cardiovascular diseases. Such intestinal imbalance could promote cholesterol accumulation [4], oxidative stress, and inflammation [5], while probiotics modulation showed protective effects in cardiovascular disease [6], implying the gut microbiota is a significant contributor to cardiovascular homeostasis.

The gut microbiome and its related metabolites have been reported to be involved in cardiovascular disease [7] since the epoch-making studies about trimethylamine-N-oxide (TMAO) [4,8]. The levels of TMAO were found closely associated with cardiovascular disease risk because TMAO enhances cholesterol accumulation in macrophages, increases cell surface expression of proatherogenic scavenger receptors [4], prolongs hypertensive effects of angiotensin II, as well as enhances platelet activation [9], which may contribute to platelet hyperresponsiveness and thrombosis. Besides, other gut microbial-host co-metabolites, such as bile acids, short-chain fatty acids, and tryptophan-related metabolites also served as essential mediators between gut microbiota and the cardiovascular system. For example, the elevated bile acids [10] and indole-sulfate [11] showed a close link with the occurrence of cardiovascular disease risk. On the contrary, the short-chain fatty acid propionate could ameliorate vascular calcification by intestinal microbiota *Akkermansia muciniphila* remodeling [12] as well as the indole-3-carboxaldehyde alleviated atherosclerosis by reducing ROS levels and inflammatory factors in endothelial cell [13]. Furthermore, the short-chain fatty acid acetate and propionate were reported to lower the

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heart rate and cardiac contractility along with blood pressure in animal models [14] or multi-center clinical trials [15], implying the role of microbial-related metabolite in the physiological regulation of the cardiovascular system.

Phenylacetylglutamine (PAGln) is a newly gut microbiome-related metabolite reported by Nemet *et al*. in a cohort with cardiovascular disease from an untargeted metabolomics profiling platform. The increased levels of PAGln were associated with major adverse cardiovascular events (MACEs) such as myocardial infarction, stroke, or death in the population [16]. Recently, a series of studies found an association between PAGln and cardiovascular diseases, such as heart failure, coronary heart disease, and atrial fibrillation. Here we reviewed the metabolic pathway of PAGln, the role of gut microbiota-dependent PAGln in several common clinical cardiovascular diseases and its possible mechanism. On the other hand, since the production of PAGln across multi-organs such as the intestine, liver, heart, and kidney, we also discussed the crosstalk among different organs of this uremic metabolite and explored possible preventive ways for cardiovascular disease by PAGln modulation. Finally, we highlighted important gaps currently in knowledge and areas requiring future investigation of PAGln in cardiovascular diseases.

Production and measurement of PAGln

The metabolic pathway of PAGln production

The PAGln comes from the essential amino acid, phenylalanine [17]. Since the human body cannot synthesize phenylalanine itself, phenylalanine is mainly dependent on food intake and is absorbed in the small intestine [18]. Previous studies have found that amino acid catabolism associated with proteolysis is significantly increased due to higher intake and consumption of cardiac energy substances in heart failure conditions [19]. In addition to its involvement in energy metabolism, the unabsorbed phenylalanine is then metabolized in the large intestine by the gut microbiota to produce phenylpyruvic acid [20] (Fig. 1). There are two distinct gut microbial pathways for conversion from phenylpyruvic acid into phenylacetic acid in the presence of *Christensenellaceae, Lachnospiraceae,* and *Ruminococcaceae* [21]. One is catalyzed by phenylpyruvate ferredoxin oxidoreductase to form phenylacetyl-CoA. The other is catalyzed by phenylpyruvate decarboxylase to form phenylacetaldehyde [22]. Further microbial colonization in germ-free mice found that the microbial *porA* gene is associated with PAGln production which favored the conversion of phenylpyruvate to phenylacetic acid [16]. Then the phenylacetic acid enters the liver through the portal system, conjugating with glutamine to form PAGln in humans and with glycine to form phenylacetylglycine (PAGly) in rodents [22].

PAGln is a normal constituent of human urine that is excreted from the kidney primarily as tubular secretion [23,24]. The PAGln clearance is greatly reduced when glomerular filtration is impaired (as measured by eGFR) [24,25] and usually, is accumulated in the plasma of uremic patients [26,27]. Such accumulation is considered as an early marker of renal dysfunction previously [28] and is applied to predict clinical endpoints in patients with chronic kidney disease [29]. Furthermore, PAGln was also known as the main product of the treatment of acute/severe hepatic encephalopathy [30,31]. The urinary PAGln can be used as a non-invasive biomarker to facilitate daily monitoring of phenylalanine metabolism in phenylketonuria patients [32] and to set up as an index for monitoring "non-invasive chemical biopsy of human liver" and is assumed to reflect liver citric acid cycle intermediates [33,34].

Measurement of PAGln

The PAGln is an amino acid derivative that can be measured by several analytical techniques including nuclear magnetic resonance (NMR), liquid chromatograph-mass spectrometer (LC-MS), and gas chromatograph-mass spectrometer (GC-MS) (Table 1).

In fact, PAGln is not a newly-discovered metabolite. Shockcor *et al*. reported that urinary PAGln is a typical uremic toxin under the existence of gut microbiota since the 1990s by the NMR platform [35]. Because the level of urinary PAGln is relatively high (34–47 μM/mM creatinine) [23], it is easy to measure based on the NMR-dominated untargeted metabolic platform previously. Fukui *et al*. identified urinary PAGln as a novel marker of interstitial cystitis [36], which indicated the disturbance of the gut microbiome in these patients. Compared to the mass spectrometer, the sensitivity of NMR platform is relatively low even though it possesses the advantage of simple sample preparation and high throughput [37,38]. Hence, it is hard to quantify the level of PAGln in plasma on the NMR platform because of its relatively lower concentration.

Several research groups tried to quantify the level of PAGln in blood plasma/serum samples with the aid of a state-of-the-art mass spectrometry platform [39] since the level of plasma PAGln is less than ten percent of that in urine. Usually, the physiologic median value of PAGln level in plasma is 0.8 μM in healthy control subjects and 2.0 μM in heart failure patients [16,40]. With the aid of the deuterated metabolite analog, the PAGln could be quantified accurately and its measurement stability was verified in the LC-MS/MS platform [40,41]. Besides, the levels of PAGln could also be measured by another

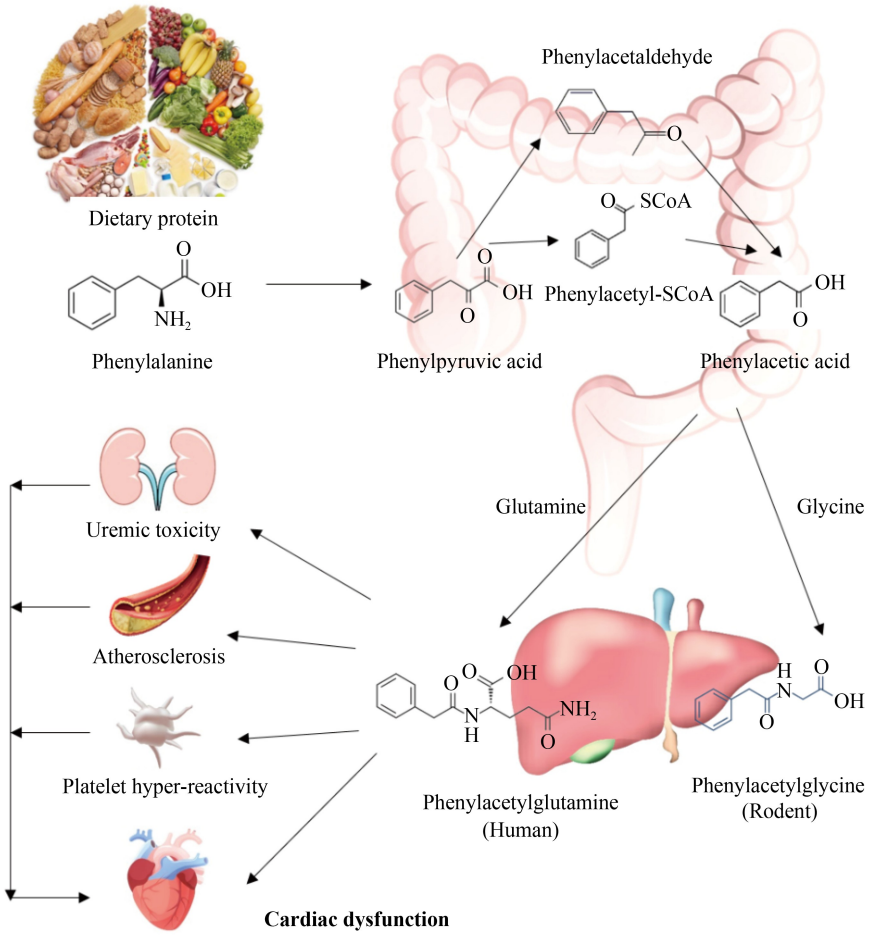

Fig. 1 The metabolic pathway of phenylacetylglutamine. The PAGln comes from the dietary protein, phenylalanine. Under the existence of gut microbiota in large intestine, the dietary phenylalanine converts into phenylpyruvic acid. The conversion of phenylpyruvic acid into phenylacetic acid could be achieved through two microbial pathways that produce two intermediates, phenylacealdehyde and phenylacetyl-CoA, respectively. The phenylacetate is converted to either phenylacetylglutamine (PAGln, dominant pathway in human) or phenyalcetylglycine (PAGly, dominant pathway in rodents) by host liver enzymes. The PAGln/PAGly take effects on cardiac dysfunction by its uremic toxicity and platelet hyperreactivity activation in cardiovascular diseases.

Table 1 Selected metabolomic approaches for PAGln quantification

Sample	Pathology	No. of subjects	Reported ConC (μmol/L)	Methodology	References
Plasma	HF	Con = 2820 HF = 1265	2.5 (0.5–14)	LC-MS/MS	[91]
Plasma	HF	Con = 1955 HF = 3152	0.87 (0.47–1.69) 1.56 (0.76–3.11)	LC-MS/MS	[40]
Plasma	CAD	Con = 35 CAD = 68	0.20–0.30	ELISA	[43]
Plasma	AF	Con = 42 AF = 92	0.18–0.30	ELISA	[44]
Plasma	Healthy rhesus monkey	$N = 5$	2.5–6	GC-MS/MS	[42]
Urine	NMR database	–	34.0 (4.5–70.0) μM/mM creatinine	^{1}H NMR	[23]
Urine	IC	Con = 10 IC = 10	47.03 (3.84–85.51) μM/mM creatinine	^{1}H NMR	[36]
Urine	Healthy rhesus monkey	$N = 5$	21.6 μM/mM creatinine	GC-MS/MS	[42]
Saliva	Healthy subjects	$N = 18$	0.04–0.08	LC-MS/MS	[46]

ConC, concentration; Con, control; HF, heart failure; CAD, coronary artery disease; IC, interstitial cystitis; AF, atrial fibrillation; LC-MS/MS, liquid chromatography with tandem mass spectrometry; GC-MS/MS, gas chromatography with tandem mass spectrometry; NMR, nuclear magnetic resonance; ELISA, enzyme linked immunosorbent assay.

analytical technique, gas chromatography mass spectrometry [42]. However, because PAGln is not a commonly volatile organic compound, this method is seldom used by other groups.

In addition to the analytical method, the ELISA measurement of PAGln has also been reported in recent studies. The levels of PAGln reported from ELISA studies [43,44] are relatively lower when compared with studies measured by the LC-MS/MS platform. It might be related to the wide coverage range and fewer exogenous interferences of LC-MS/MS, with the selective multiple-reaction monitoring capable of choosing the specific ions [45]. However, despite these technique differences, the scientific conclusion was not affected significantly when comparing the level of PAGln between the cardiovascular disease group and healthy controls. The LC-MS/MS platform takes advantage of high precision and high coverage range for PAGln measurement. That is also why the LC-MS/MS platform is the most widely used analytical tool for PAGln measurement clinically.

Except for the plasma and urine, the level of PAGln in the saliva has also been explored recently by a French group. The saliva PAGln level is approximately ten percent of its plasma concentration by LC-MS/MS platform [46]. In a small healthy volunteer cohort, Fabresse *et al*. found the PAGln level in saliva was not correlated with the PAGln level in serum. Such inconsistency might be due to the degradation by oral microbiota [47] or lack of organic anion transmembrane transport from the blood to saliva [48].

Role of PAGln in cardiovascular disease based on epidemiological evidence

In Nemet's studies, they found that the increased levels of PAGln showed prognostic value in MACEs of those patients undergoing routine cardiac evaluation. That is, the incidences of the MACEs are not specified to which kind of cardiovascular disease [16,49]. The epidemiological evidence from other different cardiovascular disease cohorts showed the diagnosis or prognostic value of PAGln in heart failure, coronary heart disease, atrial fibrillation, and atherosclerosis (Table 2).

Heart failure

Heart failure is a heterogeneous disease caused by many different etiologies [50]. Recently, the understanding of heart failure progression has been further enhanced by the aspect of "gut hypothesis" [51,52]. That is, the reduced cardiac output causes ischemic and/or edematous change in the gut, leading to translocation of the gut microbiota and increased production of circulating gut-microbiota-related metabolites and endotoxins, which in turn leads to further damage to the cardiovascular system [15,16]. As a consequence, the dynamic levels of circulating microbial-related metabolites possess the potential to reflect the role of gut microbiota in the development of heart failure [53].

Relatively few studies on PAGln and heart failure are currently available. In a prospective study of 1744 African Americans, a variety of metabolites including PAGln were screened for risk of heart failure using an untargeted metabolomics approach [54]. In a small population-based study, plasma levels of PAGln were higher in patients with heart failure-related death or re-hospitalization within one year than those in the non-event group in acute decompensated heart failure [55]. Several clinical studies based on multi-center cohorts of heart failure with reduced ejection fraction showed that elevated PAGln level is an independent risk factor for heart failure and is associated with an increased risk of cardiovascular mortality [56,57]. Considering that many of these studies were based on non-targeted methods and relatively small sample sizes, the plasma PAGln in patients with heart failure using targeted strategy LC/MS-MS in a larger cohort to date was measured in our recent

Table 2 Epidemiological or animal evidences of PAGln/PAGly associated cardiovascular diseases

Subjects	No. of patients	Study type	Sample type; assay method	Main results/findings	References
Cardiovascular disease					
Subjects with cardiac evaluation	Discovery cohort (n = 1162); Validated cohort (n = 4000)	Prospective study, 3-year follow-up	Plasma; untargeted and targeted LC-MSMS	The increased PAGln is to be associated with cardiovascular disease (CVD) and incident major adverse cardiovascular events	[16]
Subjects with cardiac evaluation	US cohort (n = 4000); EU cohort (n = 833)	Prospective study, 3-year follow-up	Plasma; LCMS/MS	Phenylalanine derived PAGln showed association with incident major adverse cardiovascular events and poorer survival risks	[49]
Heart failure					
HF patients	58 HF patients (control = 22, NYHAIII HF = 29, NYHAIV HF = 29)	Retrospective study	Plasma; LC-MS/MS	The increased PAGln were altered with different grades of chronic heart failure	[117]

(*Continued*)

Subjects	No. of patients	Study type	Sample type; assay method	Main results/findings	References
HF patients	Cohort 1: 712 HF patients (control = 2544, HF = 712). Cohort 2: 553 HF patients (control = 276, HF = 553)	Retrospective study	Plasma; LC-MS/MS	PAGln is clinically and mechanistically linked to heart failure presence and severity	[91]
HF patients	61 HF patients (HF with events = 31; HF without events = 30)	Retrospective study	Plasma; UPLC-TOFMS	Increased PAGln predicted HF events. The PAGln has better discrimination than B-type natriuretic peptide (BNP) by AUC in HF patients	[55]
HF patients	956 subjects (control = 485; HF = 471)	Retrospective study	Plasma; LCMS/MS	Elevated PAGln levels are an independent risk factor for HF and are associated with higher risk of cardiac death	[118]
HFrEF and HfpEF patients	3024 HF patients: HFpEF (*n* = 1724); HFrEF (*n* = 1300); control (*n* = 1955)	Prospective study, 2-year follow-up	Plasma; LCMS/MS	Higher plasma PAGln is associated with a higher risk of events in both HF subtype patients. PAGln provides concurrent and complementary prognostic value for NT-proBNP in HF	[40]
HFrEF with diabetes and chronic kidney diseases (CKD)	260 subjects (control = 23, HF = 48, prediabetes + HF = 83, diabetes + HF = 56, prediabetes/HF/CKD = 34, diabetes/HF/CKD = 16)	Prospective study, 5-year follow-up	Plasma; LCMS/MS	PAGln is associated with both CHF and CKD but not diabetes	[96]
Coronary artery disease					
PCI patients	*n* = 72	Retrospective study	Plasma; LCMS/MS	Plasma Phe and PAGln are valuable indices for predicting coronary in-stent restenosis	[65]
Post-PCI patients	*n* = 103	Retrospective study	Plasma; ELISA kit	Enhanced microbiota-derived PAGln synthesis-related functions and elevated plasma PAGln levels were associated with in-stent stenosis and hyperplasia in CAD	[43]
CHD patients	*n* = 686	Retrospective study	Plasma;LCMS/MS	An independent association between plasma PAG levels and the coronary atherosclerotic burden among patients with suspected CAD	[58]
Incident coronary artery disease	Total *n* = 5017: cohort 1 (*n* = 3361); cohort 2 (*n* = 880); cohort 3 (*n* = 776)	Prospective study for cohort 1	Plasma; UPLC-TOFMS	PAGln is associated with increased risk of incident coronary artery disease independently of other cardiovascular risk factors	[59]
Atherosclerosis	*n* = 316	Retrospective study	Plasma; UPLC-TOFMS	Increased PAGln was found in unexplained extreme atherosclerosis	[66]
Ischemia/reperfusion	Rodent model; *n* = 6	–	Plasma; ELISA kit	PAGly could suppress cardiomyocyte apoptosis caused by myocardial I/R injury and reduce the infarct size	[76]
Arterial stiffness	*n* = 617 women	Retrospective study	Plasma; LCMS/MS	The increased PAGln is reported in low arterial stiffness women patients and microbiome factors explained 8.3% of the variance in arterial stiffness	[61]
Cardiac arrhythmia					
Atrial fibrillation	92 AF patients: contorl = 42; AF = 92	Retrospective study	Plasma; ELISA kit	PAGln increased apoptosis, reactive oxygen species production, CaMKII and RyR2 activation and decreased cell viability	[44]
Ventricular arrhythmias	Animal model, *n* = 6	–	Plasma; LCMS/MS	PAGln increased the susceptibility of VAs in HF mice by activating the TLR4/AKT/mTOR signaling pathway	[69]

studies [40]. On the other hand, because of the different causes of main subtypes of heart failure, that is, heart failure with reduced EF (HFrEF) and heart failure with preserved EF (HFpEF), it is still unknown whether the role of PAGln in the heart failure subtypes could be different or not from previous studies. Our results confirmed that PAGln is an independent risk predictor for cardiovascular events in different subtypes of heart failure (including HFrEF and HFpEF). The level of PAGln is highly associated with deleterious cardiac function which was evaluated by the New York Heart Association class, especially for the increasing risk of cardiovascular death or hospital readmission, regardless of HFpEF and HFrEF [40]. This is the first study that manifested the role of PAGln in heart failure patients with preserved ejection fraction.

Coronary heart disease/atherosclerosis

The level of PAGln is reported to be associated with coronary heart disease/atherosclerosis patients in the Asian [58] or Western [59] population. In two independent prospective cohorts, Ottosson *et al*. found that PAGln was associated with an increased risk of coronary heart disease and was independent of other cardiovascular factors [60]. Recently the increased PAGln is also reported in lower arterial stiffness women patients. The carotid-femoral pulse wave velocity, a measure of arterial stiffness, was associated with the gut-microbiome derived PAGln and they found that the microbiome factors could explain 8.3% of the variance in arterial stiffness [61]. Yu *et al*. showed that plasma PAGln levels are not only higher in patients with ischemic stroke than in healthy subjects [62] but also associated with increased white matter hyperintensity burden in patients with acute ischemic stroke [63]. Furthermore, serum PAGln level is also elevated in patients with peripheral artery disease compared with non-peripheral artery controls [64]. When considering the coronary computed tomographic angiography together, Liu *et al*. found that the level of PAGln is independently correlated with the coronary atherosclerotic burden among patients with suspected coronary artery disease [58]. For those coronary artery patients undergoing percutaneous transluminal coronary intervention, Fu *et al*. found that the increased levels of PAGln could distinguish the coronary in-stent restenosis group from the in-stent patency in a small cohort of coronary artery disease. But it is not helpful for differentiating the in-stent hyperplasia group with a marginal statistical *P* value ($P = 0.094$), which might be due to the small sample size [65]. These results suggested that PAGln is a valuable index for predicting coronary in-stent restenosis and gut microbes may be a promising intervention target to prevent its progression. Such dysbiosis of microbial diversity and composition was confirmed by the fecal 16S rRNA sequencing in coronary artery disease patients with stent stenosis. The significant enrichment of *Roseburia, Blautia,* and *Ruminococcus* was enhanced in coronary artery disease with in-stent stenosis when compared with in-stent intimal hyperplasia patients [43].

Except for the common coronary artery disease patients, the gut-derived PAGln is also helpful for differentiating the explainable and unexplainable atherosclerosis patients. In a nested case-control study (316/3056 patients) of atherosclerosis, Bogiatze *et al*. found increased PAGln in the atherosclerosis patients with explained reasons when compared to the controls. Interestingly, for those unexplainable patients, that is, 5% with extremely low levels of known risk factors but with a very high plaque burden, the concentration of PAGln is also higher than the patients with explainable reasons. It is worth noting that there were no significant differences in PAGln precursors (phenylalanine and glutamine) among the comparable three groups (control, explainable, and unexplainable atherosclerosis patients). It implies the disturbance of the gut microbiome that contributes to the residual risk of atherosclerosis [66].

Cardiac arrhythmia

In recent decades, intricate impacts of the gut microbiome on cardiac arrhythmia have been identified as prospective approaches for its prevention, development, treatment, and prognosis. The direct evidence comes from the metagenomic and metabolomic profiling of matched atrial fibrillation patients. Except for the increased relative abundance of *porA*, the dramatic elevation in microbial diversity and overgrowth of *Ruminococcus*, *Streptococcus*, and *Enterococcus*, as well as reduction of *Faecalibacterium*, *Alistipes*, *Oscillibacter*, and *Bilophila* were detected in both fecal and serum samples in patients with atrial fibrillation [67]. A series of gut microbial-related metabolites, such as TMAO, indoxyl sulfate, and primary bile acids have also been proven to mediate molecular pathways linked to atrial fibrillation [68]. The association between newly gut-microbial related PAGln and atrial fibrillation was explored in 92 patients. Plasma PAGln levels were higher in atrial fibrillation patients and its increased levels were correlated with the left atrial diameter. The increased PAGln exerted a predictive potential of atrial fibrillation, and the combination of PAGln and atrial fibrillation score helps improve the predictive model [44].

Except for atrial fibrillation, ventricular arrhythmias are more dangerous caused by an electrical malfunction, which can drastically reduce blood flow to the rest of the body. In a thoracic aortic coarctation-induced ventricular arrhythmias model, Fu *et al*. found that PAGln deteriorated the susceptibility of ventricular arrhythmias

[69], which suggests the role of PAGln in the modulation of cardiac arrhythmia.

Potential mechanisms of PAGln in CVD

Adrenergic receptor mediated thrombosis

Along with the findings that the increased PAGln was associated with MACEs from all patients without specific cardiovascular etiology, Nemet *et al*. proposed a possible mechanism that PAGln could promote the activation of platelets and thrombosis [16]. According to multiple genetic and pharmacology studies, PAGln activates platelet responsiveness and thrombosis potential through G-protein coupled receptors (including α2A, α2B, and β2-adrenergic receptors) in both cellular and animal models (Fig. 2). In the $FeCl_3$-induced carotid artery injury model, intraperitoneal injection of PAGln/PAGly (the PAGln analog in rodent mice) promotes platelet thrombus formation and reduces the time to cessation, while the use of β-blocker carvedilol reverses this pro-thrombotic effect [16]. Since most previous studies focused on the correlation between the level of PAGln and cardiovascular diseases in the view of epidemiology, the potential mechanisms are relatively few. This work by Nemet *et al*. not only confirms that PAGln is a new intestinal flora-dependent metabolite associated with cardiovascular disease but also leads the study of the association of intestinal flora in the field of cardiovascular disease to causal-orientated research [70].

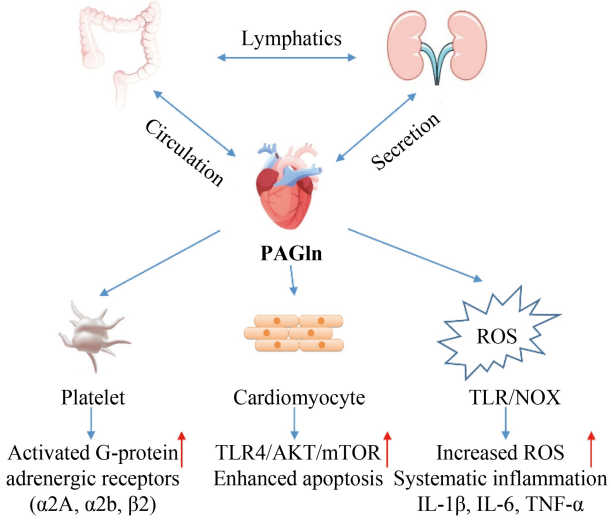

Fig. 2 Effects of PAGln on the cardiovascular system and its different mechanisms. The inter-organ crosstalk among the intestine, heart, and kidney is critical for PAGln production in maintaining homeostasis. The increased PAGln could activate the G-protein adrenergic receptors, enhance apoptosis through the TLR4/AKT/mTOR signal pathway, and increase systematic inflammation. NOX, NADPH oxidase; ROS, reactive oxygen species; TLR4, Toll-like receptor 4; AKT, protein kinase; mTOR, mammalian target of rapamycin; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tumor necrosis factor α; PAGln, phenylacetylglutamine.

It is worth noting that in recent studies, the interaction effects between the level of PAGln and the use of β-blocker were not significant in a relatively large heart failure population [40]. For comparing the effects of β-blocker in the different quartiles of PAGln level to those without the usage of β-blocker, the Kaplan–Meier plots show that the usage of β-blocker takes benefits for heart failure patients, regardless of each stratified PAGln quartile. That means the presence or absence of β-blocker did not affect the relationship between PAGln and heart failure prognosis in the view of epidemiology [40]. These results also indicate that there might be more complex underlying mechanisms except for the adrenergic receptor-mediated thrombosis in the disease development of heart failure.

Inflammatory responses

Previously, the classical microbiome derived metabolite TMAO has been found to activate the NLR family pyrin domain containing 3 inflammasome-related inflammatory pathways [71,72] and exacerbate cardiac fibrosis in the carotid artery ligation mouse model [72,73]. The other gut microbial derived indoxyl sulfate increased the expression of pro-inflammatory and pro-fibrotic signaling molecules and oxidative stress in atrial fibrillation patients [74], which revealed the role of microbial related metabolites in promoting inflammatory responses in cardiovascular diseases. Here the administration of PAGln was also reported to deteriorate myocardial inflammatory cell infiltration and increase the inflammatory response by activating Toll-like receptor 4 and its downstream protein kinase and mammalian target of the rapamycin signal pathway [69], implying the activated inflammatory responses by PAGln. Actually, the inflammatory response index was found promoted in the thoracic aortic coarctation induced heart failure mouse model, as well as in the heart failure patients [75], but the gut microbial related PAGln could deteriorate such inflammatory responses. Such elevated inflammatory indices (such as IL-1β and IL-6) induced by PAGln were also confirmed in isoprenaline stimulated neonatal rat cardiac myocytes [40]. These above results imply that the increased inflammatory response and activated immune systems induced by PAGln contribute to the disease progression of cardiovascular disease.

Protective effects of PAGln

Even elevated PAGln was found to increase the predictive value of cardiovascular event risk in many cardiovascular clinical cohorts. However, there are also some preliminary basic studies suggesting potential

protective effects of PAGln/PAGly under certain conditions. For example, Xu *et al*. found that the analog of PAGln, PAGly, activates the Gαi/PI3K/AKT anti-apoptotic signaling pathway via B2AR and reduces hypoxia/reoxygenation injury in neonatal mouse cardiomyocytes [76]. Administration of a moderate dosage of PAGly to mice with hypoxia/reoxygenation injury reduced the size of the myocardial infarct zone, but high dosage of PAGln failed to have this effect, which may be related to the heterogeneity within the high-dosage group [76]. Another *in vitro* study demonstrated the anti-inflammatory properties of PAGln in mouse spleen cells and peritoneal cavity cells [77] by increasing ROS production, reducing cell activity, and increasing apoptosis in HL-1 cells [78]. Therefore, more rigorous clinical studies and deeper mechanistic studies are necessary to explore the relationship between PAGln and cardiovascular diseases in the future.

Inter-organ communication in PAGln uremic toxicity

As previously mentioned, the PAGln is produced from protein metabolism and influenced by colonic microbiota and enterohepatic circulation [49]. This gut-derived low molecular weight uremic toxin [79] is removed by glomerular filtration in the kidney. Generally, the kidney-intestinal cross-talk involves intestinal epithelial damage, dysbiosis, and generation of uremic toxins. Recently, Zhong *et al*. revealed that kidney injury expands the intestinal lymphatics, which is a route for transporting uremic toxins and alters the composition of mesenteric lymph in kidney disease [80].

On the other hand, these small molecules could be transported into circulation across the barrier in the intestine, the heart, and the kidney and play roles in inter-organ and inter-organism communication [81]. From a perspective of the remote sensing and signaling theory of these gut derived uremic toxins, Glorieux *et al*. raised a point that these uremic toxins including PAGln, are critical for the maintenance of homeostasis along the gut–heart–kidney axis and for responding to homeostatic perturbations [81]. The remote sensing and signaling theory has been used to understand the roles of transporters, enzymes, and kinases in inter-organ and inter-organism communication via small endogenous molecules [82,83]. For example, these uremic toxins including PAGln have been demonstrated as fibrogenic and prohypertrophic effects [84] as well as aortic calcification, endothelial cell dysfunction, and tubular damage in the kidneys, indicating their roles on the heart, vessels, and kidneys. Furthermore, this inter-organ crosstalk was also confirmed by gastrointestinal and hepatic involvement in heart failure patients [85]. In our previous studies, the increased level of PAGln showed a close correlation with creatinine in heart failure patients [40]. The close link between gut–heart–kidney crosstalk reminds us not to put this gut-derived metabolite PAGln as a biomarker only specific for cardiovascular or kidney diseases. The disturbance of PAGln could be modulated from each view of the gut, heart, and kidney. In other words, even if the potential mechanism was poorly revealed, those individuals with relatively normal renal function or normal intestinal activity might be at a higher risk of cardiovascular events due to the elevated PAGln and hence should be given sufficient attention. On the contrary, as Drs. Spence and Urquhart suggested, the high cardiovascular disease risk in chronic kidney disease patients also requires consideration of therapies due to this kind of gut-derived uremic metabolite [86].

Gaps in knowledge and future directions

Comparison between PAGln and classical biomarkers in cardiovascular diseases

Since PAGln has proven to be a potential biomarker that possesses the value in diagnosis and prognosis of cardiovascular disease, its comparison with the traditional biomarker could not be ignored. The highly sensitive cardiac troponin (hs-cTnI) and N-terminal proB-type natriuretic peptide (NT-proBNP) are classical biomarkers in managing ischemic cardiomyopathy [87,88] and heart failure [89]. The level of NT-proBNP takes the priority that is recommended in the diagnosis and prognosis of heart failure by the American Heart Association [89]. Due to the recruited sample numbers of the heart failure cohort being small and the lack of follow-up records, previous studies related to the interaction between PAGln and NT-proBNP are relatively few. On the other hand, most of the previous studies focused on typical heart failure whose ejection fraction is less than 40% (HFrEF). In a small sample size cohort, Tang suggested that four metabolites including PAGln could increase the predictive value of BNP in heart failure patients [55]. Recently, in our group including 3152 heart failure patients, we stratified the heart failure patients based on the levels of PAGln and NT-proBNP, respectively. The group with the highest quartile and PAGln and NT-proBNP demonstrated the highest risks for getting major adverse cardiac events, compared to a reference group with the lowest quartile of PAGln and NT-proBNP, confirming the joint effects of PAGln and NT-proBNP in a larger population [40]. Besides, when the reference was set to group with its first quartile of NT-proBNP, the PAGln showed predictive value for a group with lower (or normal) NT-proBNP, especially in heart failure patients with preserved ejection fraction, implying the complementary effects of PAGln to the classical index NT-proBNP. Our studies got editorial comments for highlighting their potential application in the prognostic value of cardiovascular death or hospital

readmission in HFpEF patients [90].

The consequent question is why the PAGln possesses more predictive value in heart failure with normal NT-proBNP. The main causes of HFpEF are cardiac metabolic factors, such as higher body mass index (BMI), hypertension, diabetes, and obesity. The BMI index was inversely associated with the level of PAGln not only in total HF patients but also in different subtypes of HF, i.e., HFpEF and HFrEF in our previous studies. Such results agreed with findings from a recent study by Romano *et al*. [91] and urinary PAGln in the human adiposity population [92]. Considering that obesity is the main cause of HFpEF, it was believed that low BNP levels were more commonly seen in HFpEF because of two possible reasons. The first is that androgens produced by adipose tissue could inhibit the expression of the natriuretic peptide precursor B gene, resulting in reduced release of BNP [93]. On the other hand, it may be related to the abundance expression of natriuretic peptide receptors on adipocytes, which increases the clearance of BNP and leads to a decrease in BNP levels [94]. Therefore, obesity should be an important mediator of heart failure, especially for HFpEF patients.

Furthermore, except for BMI, the increased level of PAGln is also related to the level of glucose [95]. The relationship between PAGln and T2DM and its complications has been corroborated by a series of subsequent modest clinical studies [96]. PAGln was positively correlated with the severity of T2DM after adjusting for sex and age [41]. Among diabetic patients, those who progressed to distal symmetric polyneuropathy [97], diabetic nephropathy [98], and diabetic retinopathy [99] had even further elevated levels of PAGln in the circulation. In contrast, Hua *et al*. reported the increased PAGln associated with chronic kidney diseases and heart failure, but not diabetes in their cohort [96]. These studies suggested that metabolic syndrome could not be ignored when evaluating the role of PAGln in cardiovascular diseases.

Modulation of PAGln

Usually, the classical intervention ways that modulate the gut ecosystem include diet [100,101], microbial modification (such as facel microbial transplantation and probiotics modulation), and specific inhibitors [102,103]. For example, a high-protein diet did not affect serum TMAO levels whereas PAGln was significantly elevated in an elderly woman cohort, which suggests that the effect of dietary intake of different components on TMAO/PAGln in human circulation may differ [104]. A recent study shows that PAGln may be involved in the regulation of blood pressure by the Dietary Approaches to Stop Hypertension Diet (DASH) [105]. That is because the dietary fibers in these diets which are regarded as common prebiotics, were not absorbed by the upper gastrointestinal tract and utilized by colonic microbiota. Kaye *et al*. found that dietary deficiency of prebiotic fiber led to increased blood pressure and pathological myocardial remodeling in mouse models [106], which could be reversed by high fiber diet treatment [107] or green tea containing the precursor of PAGln [108].

Tang *et al*. provided alternative ideas to modulate the gut microbiota directly. For example, the antibiotic-treated mice had significantly increased mortality after infarction, and probiotic supplementation or fecal transplantation was able to repair damaged myocardium via SCFAs [5]. The use of antibiotics by modulating gut microbiota for the treatment of cardiovascular diseases has become a major research interest. The use of antibiotics in rats with myocardial infarction was able to modulate microbial metabolites and reduce the size of the infarction part [109]. In animal models of HF, antibiotic administration reduced plasma TMAO levels, attenuated myocardial hypertrophy, and fibrosis [110], and exerted a protective effect [111]. However, the usage of antibiotics has to be carefully considered, because the gut microbiota is involved in normal physiologic processes, and its potential for new dysbiosis and resistance is an unavoidable problem [112]. Beyond this, a 10-year CLARICOR RCT study also showed that clarithromycin administration in patients with stable heart disease increased cardiovascular mortality [113].

Thirdly, the specific inhibitors, such as the choline analog (3,3-dimethylbutanol [114] or fluoromethycholine [103]) for inhibiting choline trimethylamine lyase activity and β-blocker carvedilol for inhibiting the adrenergic receptor in PAGln production, were used in previous studies [16]. However, further clinical application still needs to be carried out based on the safety and reliability of human studies. Besides, based on the uremic toxicity of PAGln, Fatani *et al*. found that hemodialysis could be a useful tool for lowering the levels of PAGln in those patients with high uremic metabolites. But in this case, the extra fiber supplementation does not modulate the microbial-generated uremic molecules in a randomized controlled trial [115].

In a word, even though the close association of PAGln in cardiovascular diseases, as well as the dietary intervention, showed promising application in the regulation of microbial metabolites, there is still a lack of specific way or clear dietary guideline for lowering the level of this uremic toxin metabolite [116]. Consequently, the modulation based on the level of PAGln remains a long way off.

Conclusions

The new microbial metabolite PAGln has provided ideas for the study of the relationship between gut microbiota

and cardiovascular diseases. With the development of metabolomics and metagenomics, the microbial metabolites not only serve as biomarkers to help clinicians identify high-risk cardiovascular patients for timely prevention and treatment but also provide promising targets for interventions based on the existing treatment system, such as precise dietary interventions, the use of antibiotics and probiotics.

Considering the complexity of the gut microbiota and its associated metabolites, there are still a lot of gaps that need to be filled in the current stage of research. The current findings lack more evidence from multi-center clinical trials, especially for interventional studies on the regulation of gut microbiota and metabolites. Secondly, the specific mechanisms by which microbial metabolites affect cardiovascular disease remain to be elucidated. There is also a lack of clear dietary or microbial guidelines associated with PAGln for the treatment of cardiovascular diseases. Furthermore, the cross-talk of this uremic toxicity among multi-organs in cardiovascular or kidney disease needs further investigation. The collaboration of public health scientists, biologists, and clinicians is the only way to overcome these challenges, and we expect that research on PAGln and its related microbial metabolite will make a greater contribution to the prevention and treatment of cardiovascular diseases.

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Compliance with ethics guidelines

Yaonan Song, Haoran Wei, Zhitong Zhou, Huiqing Wang, Weijian Hang, Junfang Wu, and Dao Wen Wang declare no conflicts of interest. This article does not involve a research protocol requiring approval by a relevant institutional review board or ethics committee.

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