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High Betaine and Dynamic Increase of Betaine Levels Are Both Associated With Poor Prognosis of Patients With Pulmonary Hypertension

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Background and Objective: The association between plasma betaine levels and cardiovascular diseases (CVDs) has been revealed except for pulmonary hypertension (PH). In this study, we aimed to explore the role of betaine in patients with PH.

Methods: Inpatients with PH at Fuwai Hospital were enrolled after excluding relative comorbidities. Each patient received at least one follow-up through a clinical visit, and the fasting blood was obtained both at the first and second hospitalization for betaine detection. The primary endpoint was defined as composite outcome events and the mean duration was 14.3 (6.9, 21.3) months. The associations of betaine and changes of betaine (Δbetaine) with disease severity and prognosis were explored.

Results: Finally, a total of 216 patients with PH were included and the medians for betaine plasma levels in the total patients group, low betaine, and high betaine groups were 49.8 (39.0, 68.3) μM, 39.0 (33.5, 44.7) μM, and 68.1 (57.8, 88.7) μM, respectively. High betaine was associated with poor World Health Organization Functional Class (WHO-FC), increased N-terminal pro-brain natriuretic peptide (NT-proBNP), low tricuspid annular plane systolic excursion (TAPSE), and cardiac output index even after adjusting for confounders. Patients with high betaine were over twice the risk to receive the poor prognosis than those with a low level [hazard ratio (HR) = 2.080, (95% CI: 1.033–4.188)]. Moreover, the decrease of betaine level after further treatment was positively

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correlated to ΔNT-proBNP indicating Δbetaine might be an effector of disease severity, and dynamic increase of betaine was also associated with poor prognosis in PH.

Conclusion: Betaine was associated with disease severity and might be an effector in PH. Patients with increased levels or with dynamic rise of betaine heralded a poor prognosis.

Keywords: metabolites, betaine, pulmonary hypertension, severity, prognosis, Δbetaine

INTRODUCTION

Pulmonary hypertension (PH), classified into five categories according to different etiology, is a kind of progressive cardiovascular disease (CVD) that results in heart failure and death eventually (1). Although the knowledge on PH pathogenesis and treatment has improved in the past decade, it is still regarded as an extremely complex disease that required comprehensive and time-consuming inspections and evaluations, and the survival of the patient is still worrying. Nowadays, how to better manage patients to improve their prognosis is a tremendous challenge.

Facing this plateau, the exploration of serological biomarkers in PH is a promising way for effective disease management (1). However, the well-recognized serological biomarker is lacking except for brain natriuretic peptide (BNP) and N-terminal pro-BNP (NT-proBNP) up to date. A growing body of literature recently implicates metabolites that include betaine, choline, and trimethylamine N-oxide in CVD risk (2–6). Betaine was obtained from food sources, such as grain products, vegetables, red meat, eggs, and fish (7), or synthesized *de novo* from the irreversible oxidation of choline *via* betaine aldehyde dehydrogenase and choline dehydrogenase predominantly in the liver, a process under homeostatic control in healthy humans (8). It has been suggested that high betaine levels may exert the prognostic value of major adverse cardiovascular event risk in table cardiac subjects who underwent elective diagnostic coronary angiography (9) and high dietary betaine intake was associated with an increased risk of incident coronary heart disease (10). Similar results were also reported in patients with diabetes mellitus (11).

To our best knowledge, the role of betaine in PH has never been investigated. Here, we aimed to investigate the association of plasma betaine concentration and disease severity of patients with PH and to examine whether betaine could be served as a biomarker in clinical outcomes. Moreover, we also preliminarily explored the value of dynamic changes of betaine (Δbetaine) in PH.

MATERIALS AND METHODS

This is a clinical study designed to evaluate the association between betaine levels and PH. The study was approved by the Ethics Committees of Fuwai Hospital and adhered to the Declaration of Helsinki. All patients were provided written informed consent.

Study Population and Clinical Data Collection

Pulmonary hypertension was diagnosed as mean pulmonary atrial pressure (mPAP) ≥25 mmHg by right heart catheterization (RHC) in this study. PH inpatients at Fuwai Hospital Pulmonary Vascular Word in China from March 2019 to April 2020 were enrolled. Exclusion criteria included (1) patients who were diagnosed as connective tissue disease-related PH or had the immune disease; (2) patients with acute coronary syndromes, active infection, malignancy, congestive heart failure, and diabetes; (3) incomplete clinical data; and (4) patients without rehospitalization. Clinical data that include demographic characteristics, World Health Organization Functional Class (WHO-FC), laboratory parameters, echocardiography, exercise capacity, and hemodynamics were collected in this study.

Follow-Up and Study Endpoint

Each patient received at least one follow-up through hospitalization visit and the fasting blood was obtained both at the first and second hospitalization for betaine and other clinical indicators detection. When exploring the association between betaine levels at baseline and clinical outcome, the follow-up duration was defined as the time from the first hospitalization to the occurrence of outcome or the end of follow-up. The mean duration was 14.3 (6.9, 21.3) months. In the analysis of Δbetaine, the follow-up duration started from the second hospitalization and the mean duration was 9.9 (2.7, 15.3) months.

The study endpoint was defined as composite outcome events that include death, rehospitalization due to heart failure, escalation of targeted medication due to the disease condition, deterioration of PH that includes worsening symptoms, higher WHO-FC compared with baseline, or at least 15% decreased 6-min walk distance (6MWD) from baseline (12).

Quantification of Betaine

Collecting 5 ml of blood samples at fasting and centrifuging for 10 min at a speed of 3,000 rpm. The supernatants were obtained and stored at −80°C. After thawing, 20 μl supernatants were aliquoted to a 1.5-ml tube and mixed with 80 μl of 5 μM internal standard composed of d9-metabolites in methanol. Protein in the samples was precipitated by vortexing for 1 min. Next, the samples were centrifuged at 20,000 *g* at 4°C for 10 min. In order to obtain the precise concentration of the analytes, a standard curve was performed using 20 μl of various concentration standards (0–100 μM) processed in parallel. The coefficient of determination (R^2) reached 0.99 in standard

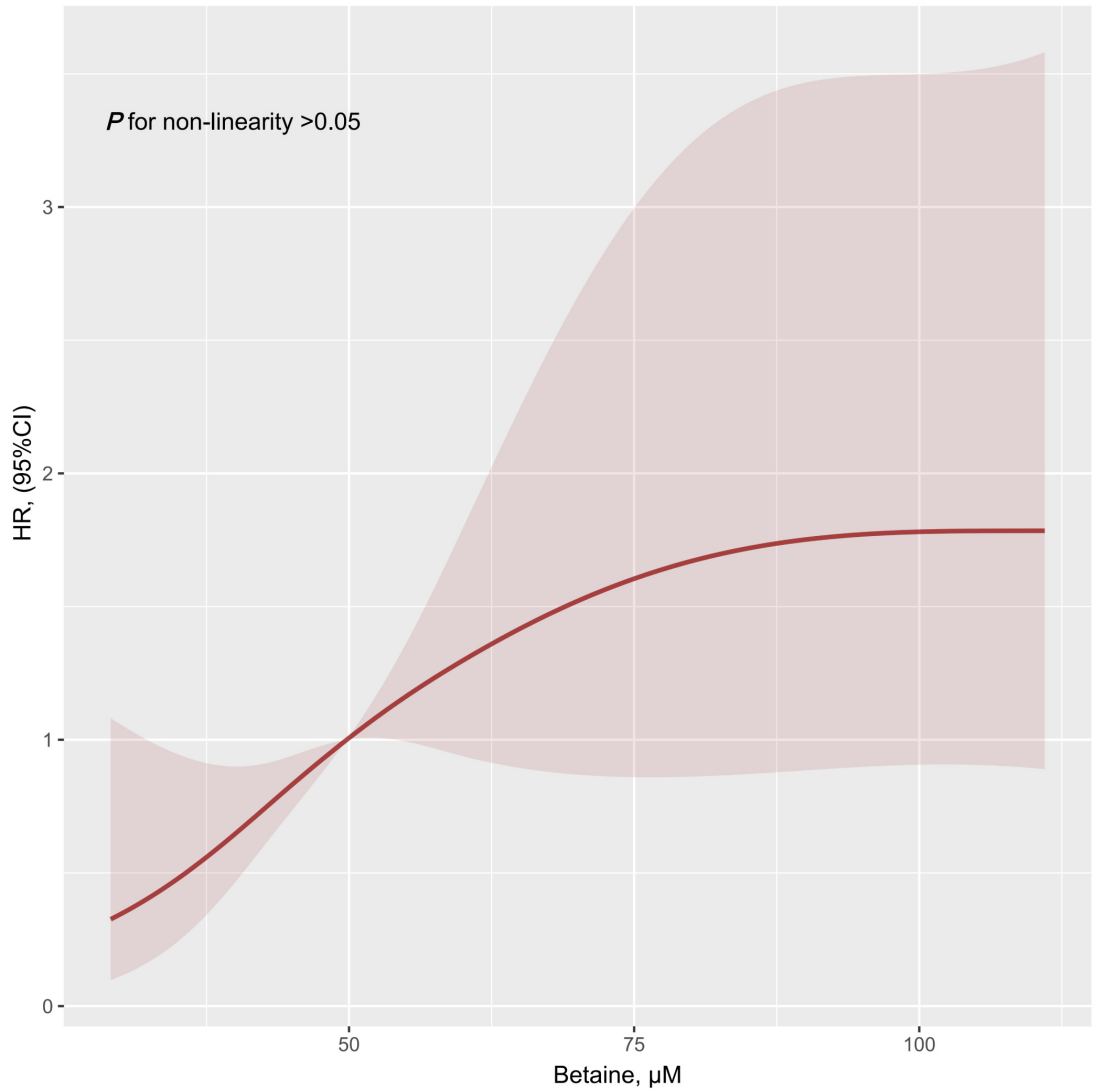

FIGURE 1 | Restricted cubic spline result of plasma betaine levels in relation to hazard ratio for the risk of clinical outcomes. Dark red line with 95% CI shaded in light red. HR, hazard ratio; CI, confidence interval.

curves were acceptable. Supernatants (70 μl) were analyzed by injecting them onto a silica column using an LC-20AD Shimadzu pump system at a flow rate of 0.5 ml/min, SIL-20AXR Autosampler interfaced with an API 5500Q-TRAP mass spectrometer. A discontinuous gradient was generated to resolve the analytes by mixing solvent A (0.1% propanoic acid in water) with solvent B (0.1% acetic acid in methanol) at different ratios. Analytes were monitored using electrospray ionization in positive-ion mode with multiple reactions monitoring of precursor. Three quality-control samples with different betaine concentrations were measured every 20 samples.

Statistical Analysis

A restricted cubic spline was used to explore the linear or non-linear relationship between betaine and clinical outcome. Student's *t*-test or Wilcoxon rank sum test for continuous variables and χ^2 test for categorical variables were used to examine the difference between groups. Paired-samples *t*-tests or paired Wilcoxon rank sum test was used to compare the changes between first and second hospitalization. Spearman's correlation (2-tailed), univariate or multivariate logistics were used to determine correlations between betaine and clinical markers of disease severity. Spearman's correlation (2-tailed) was also utilized for exploring the relation between Δbetaine and changes in the clinical indicator. Kaplan–Meier (KM) analysis and Cox proportional hazards regression were used for determining hazard ratios (HRs) and 95% confidence intervals (CIs). A two-sided $p < 0.05$ was considered statistically significant. Analyses performed in this study used R 2.8.0 (Vienna, Austria), SPSS (version 23; IBM Corp.) and GraphPad (GraphPad Software, Inc).

TABLE 1 | Characteristics of patients stratified by 50th percentile of betaine.

Variables	Total patients (N = 216)	Low betaine (N = 108)	High betaine (N = 108)	*p* value
Age, years	36 (27, 54)	32 (25, 46)	43 (30, 56)	0.001
Female sex, n (%)	141 (65.3)	79 (73.1)	62 (57.4)	0.022
BMI, kg/m^2	22.0 ± 3.9	21.5 ± 4.2	22.6 ± 3.4	0.067
WHO-FC, n (%)				
I–II	132 (61.1)	76 (70.4)	56 (51.9)	0.008
III–IV	84 (38.9)	32 (29.6)	52 (48.1)	0.008
Laboratories				
Betaine, μM	49.8 (39.0, 68.3)	39.0 (33.5, 44.7)	68.1 (57.8, 88.7)	<0.001
NT-proBNP, pg/ml	446.3 (144.3, 1563.0)	304.0 (127.0, 659.8)	821.6 (194.5, 2328.5)	<0.001
Albumin, g	42.3 ± 4.8	42.8 ± 4.5	41.8 ± 5.1	0.203
Triglycerides	1.1 (0.8, 1.5)	1.1 (0.8, 1.5)	1.1 (0.8, 1.5)	0.687
Total cholesterol, mM	4.2 ± 1.1	4.2 ± 1.1	4.1 ± 1.1	0.975
Creatinine, μM	75.0 (65.5, 90.0)	71.0 (62.4, 81.9)	82.3 (71.0, 95.0)	<0.001
Echocardiography				
LVEF, %	65.0 (60.0, 70.0)	65.0 (60.0, 69.3)	65.0 (60.0, 70.0)	0.533
RVD, mm	32.0 (27.0, 37.0)	30.0 (26.8, 36.0)	33.0 (27.5, 38.0)	0.04
TAPSE, mm	16.5 (14.0, 18.0)	18.0 (15.0, 19.0)	15.0 (13.0, 18.0)	0.005
Exercise capacity				
PeakVO_2, mL/min/kg	47.4 ± 15.3	15.0 ± 4.1	14.1 ± 3.7	0.249
VO_2%	1.5 ± 0.4	1.5 ± 0.4	1.4 ± 0.3	0.045
6MWD, m	422.3 ± 96.5	426.4 ± 86.8	417.3 ± 107.5	0.096
Hemodynamics				
mRAP, mmHg	6.0 (4.0, 8.3)	6.0 (3.5, 8.5)	7.0 (4.0, 8.5)	0.538
mPAP, mmHg	57.0 (47.0, 70.0)	57.5 (46.8, 70.5)	56.5 (47.0, 70.8)	0.726
Cardiac output index, L/min*m^2	3.2 ± 1.0	3.4 ± 1.1	2.9 ± 0.9	0.002
PAWP, mmHg	8.0 (6.0, 11.0)	8.0 (6.0, 11.0)	9.0 (6.0, 11.0)	0.452
PVR, WU	6.7 (5.0, 11.2)	6.8 (5.0, 10.0)	6.6 (4.9, 11.7)	0.861

Betaine represents plasma betaine concentrations and patients were stratified into low betaine and high betaine groups by 50th percentile of betaine (49.8 μM). BMI, body mass index; WHO-FC, World Health Organization Function Class; NT-proBNP, N-terminal pro-brain natriuretic peptide; LVEF, left ventricular ejection fraction; RVD, right ventricular diameter; TAPSE, tricuspid annular plane systolic excursion; 6MWD, 6-min walk distance; mRAP, mean right atrial pressure; mPAP, mean pulmonary atrial pressure; PAWP, pulmonary arterial wedge pressure; PVR, pulmonary vascular resistance.

RESULTS

Population Characteristics at Baseline

Finally, a total of 216 patients with PH, 140 pulmonary arterial hypertension, 61 chronic thromboembolic PH, 12 PH with multifactorial mechanisms, and 3 PH due to hypoxia, were included in this study. During the follow-up duration, 159 patients survived without clinical worsening, while 12 patients were rehospitalized for progression of PH or heart failure, 16 patients for escalation of targeted medication, and 5 patients were died.

The result of the restricted cubic spline showed the linear relationship of betaine in this study (non-linear, $p = 0.100$, **Figure 1**). Patients with PH were stratified into low betaine and high betaine groups by 50th percentile of betaine and the basic characteristics are shown in **Table 1**. The medians (interquartile ranges) for betaine plasma levels in the total patients group, low betaine, and high betaine groups were 49.8 (39.0, 68.3) μM, 39.0 (33.5, 44.7) μM, and 68.1 (57.8, 88.7) μM, respectively. Patients in the high betaine group were elder and had a higher proportion of men than the low betaine group. Moreover, patients with high betaine received worse WHO-FC, higher NT-proBNP, larger right ventricular diameter (RVD), lower tricuspid annular plane systolic excursion (TAPSE), and cardiac output index than those with low betaine plasma levels (**Supplementary Figure 1**).

Correlation Between Betaine and Disease Severity

Supplementary Table 1 shows the correlations between betaine and clinical indicators. Following adjustments for confounders that include age, sex, body mass index (BMI; **Supplementary Table 2**), increased betaine was also associated with WHO-FC [odds ratio (OR) = 2.349, (95% CI: 1.241–4.448), $p < 0.009$], NT-proBNP [OR = 1.993, (95% CI: 1.026–3.870), $p = 0.042$], TAPSE [OR = 2.026, (95% CI: 1.087–3.779), $p = 0.026$], and cardiac output index [OR = 2.390 (95% CI: 1.087–5.255), $p = 0.030$].

High Betaine Was Associated With Poor Prognosis of Patients With PH

Kaplan–Meier analysis indicated the evidence that increased betaine level was associated with poor prognosis of patients with PH (**Figure 2**, $p = 0.001$). Univariate Cox regression analysis was performed, which is shown in **Supplementary Table 3**. After adjusting for confounders that include sex, WHO-FC, creatinine, blood urea nitrogen, and cardiac output index, multivariate Cox analysis revealed that high betaine was still correlated to poor clinical outcome among patients with PH [HR = 2.080, (95% CI: 1.033–4.188), $p = 0.040$, **Table 2**].

Betaine Was Responsive to Clinical Outcome

In this section, due to the undetectable betaine levels of blood samples collected in the second hospitalization, 13 patients with PH were excluded. During follow-up, patients received relative therapy, which was recommended by the 2015 European Society of Cardiology PH guideline (1). Here, Δbetaine and ΔNT-proBNP were calculated as the value at the second admission visit minus the baseline value.

Betaine was decreased responsively among total patients [Δbetaine = −5.9 (−15.7, 7.3) μM, $p < 0.001$] positively with the decline of NT-proBNP (r = 0.25, $p < 0.001$, **Figure 3**). To further explore the association between Δbetaine and clinical outcomes, KM analysis was utilized and the result showed that Δbetaine >0 also indicated a poor prognosis of patients with PH (**Figure 4**).

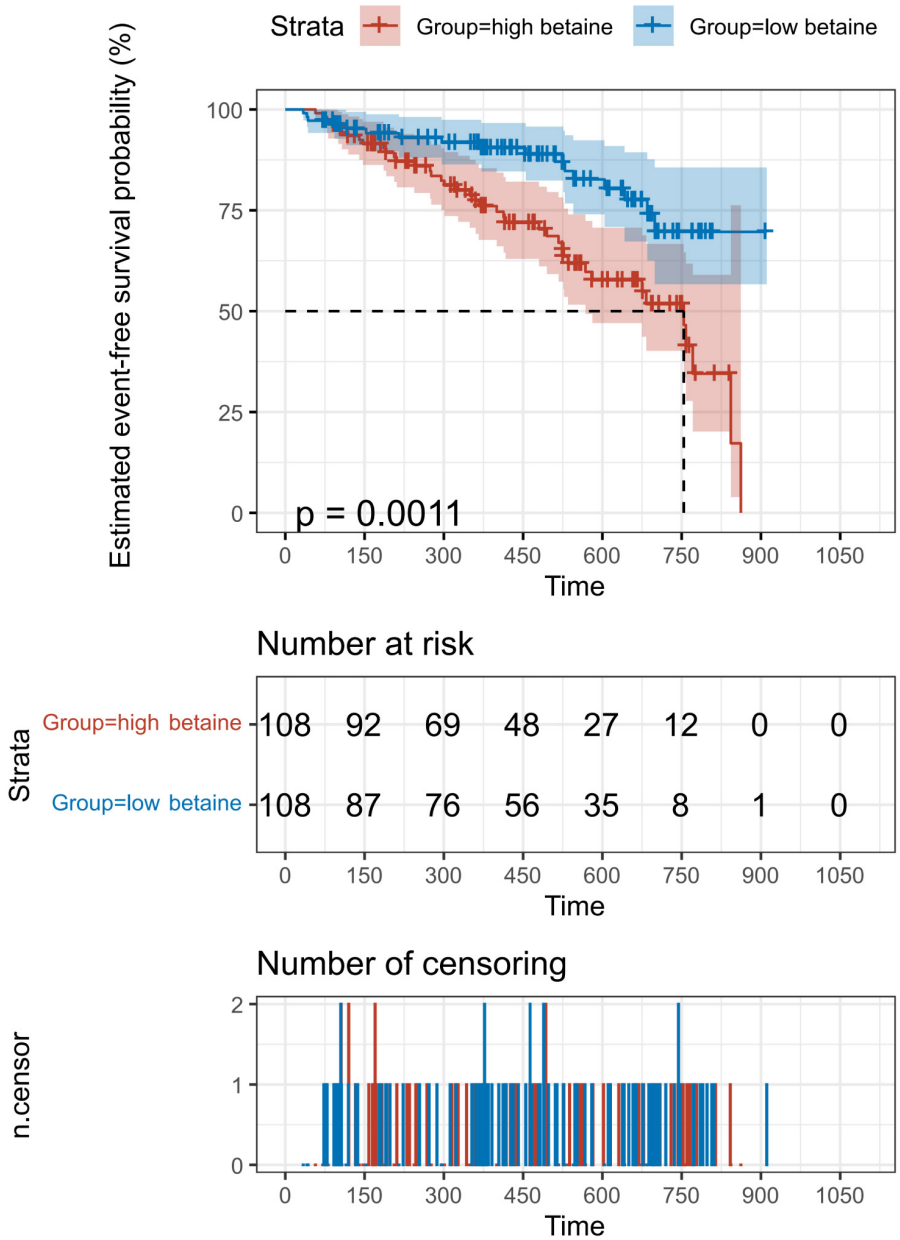

FIGURE 2 | Kaplan–Meier (KM) analysis for the incidence of composite outcome events. Patients were divided into two groups according to the 50th percentile of plasma betaine concentrations (49.8 μM) at baseline. A total of 216 patients with PH were included for exploration. Value of *p* calculated by the log-rank test.

DISCUSSION

Pulmonary hypertension is an under-recognized global health concern, and the general treatment of PH predominantly depends on the type and severity and the patient's symptoms (13). Metabolomics provides the measurement of various metabolites in human samples, which holds great promise for the discovery of pathways linked to disease processes in clinical research (14). Researchers had observed associations between betaine with the prevalence of type 2 diabetes mellitus (15). Additionally, systemic levels of choline and betaine, two closely connected metabolites in the dietary lipid phosphatidylcholine, were also reported to be independently associated with the prevalence of CVDs (14). Moreover, a number of studies have reported increased concentration of betaine associated with the increased incidence of acute coronary syndromes and increased blood lipid concentrations (9, 16, 17). These studies suggest a potential link between betaine with type 2 diabetes mellitus and CVDs. However, less is known about the role of betaine in PH. In this study, we conducted a clinical study to assess the associations between plasma betaine concentration with severity and prognosis of patients with PH.

TABLE 2 | Multivariate Cox analysis of plasma betaine levels and clinical outcomes.

Variable	HR	95% CI	*p*
Betaine (categorical variable)	2.080	1.033–4.188	0.040
Sex, female	0.801	0.354–1.812	0.595
WHO-FC	1.894	1.060–3.383	0.031
Creatinine, Mm	1.027	1.002–1.052	0.037
BUN, Mm	0.845	0.685–1.042	0.116
Cardiac output index, L/(min/m^2)	0.716	0.472–1.086	0.116

Betaine represents plasma betaine concentrations. Plasma betaine levels were put into the model as a categorical variable bounded by 50th percentile (49.8 μM). WHO-FC, World Health Organization Function Class; BUN, blood urea nitrogen.

The current study's 50th percentile of plasma betaine concentration was 49.8 mmol/L (interquartile range, 39.0–68.3), which was comparable with two cohorts of patients with CVDs (9, 18). Based on this baseline, we divided the study cohort into low and high betaine groups. Several key findings were noted in the baseline and correlation analysis. First, plasma betaine level was higher in older patients, and patients with high betaine were characterized by poor WHO-FC classification, higher NT-proBNP levels, larger RVD, and lower TAPSE and cardiac output index compared to those with low levels. Second, plasma betaine level was correlated positively with age, NT-proBNP, creatinine, and WHO-FC, and negatively with TAPSE, peak VO_2, VO_2%, and cardiac output index, of which WHO-FC, NT-proBNP, TAPSE, and cardiac output index remained statistically significant after adjusting for confounding factors. These correlating variables from the laboratory tests, echocardiography, and hemodynamics were all consensus-based criteria that have been identified to evaluate the severity and the risk stratification of patients with PH (1, 19). The worsening WHO-FC is not only a strong predictor of follow-up survival but also an alarming indicator of disease progression. NT-proBNP is not specific for PH but remains the biomarker in the routine practice to provide information of myocardial stress at the time of diagnosis and follow-up assessments, which is also regarded as a strong prognostic predictor in PH (20, 21). The cardiac output index assessed by RHC and the TAPSE measured by non-invasive echocardiogram are both robust reflections of heart function and are commonly used as indicators for severity assessment (1). Moreover, high levels of betaine remained robust to predict increased adverse prognosis regardless of potential confounders. Together, these results indicated that the plasma betaine level might have a positive correlation with PH severity in our study cohort. We had noticed no statistical differences in 6MWD and peakVO_2 between groups were explored. This might attribute to the fact that severe patients with PH did not receive 6MWD and cardiopulmonary exercise tests, which narrowed the actual gap between the two groups.

To better understand the prognostic role of betaine in PH treatment, we conducted an analysis with follow-up ascertainment of primary endpoints in patients who received recommended therapies. Here, we found that both low plasma betaine and dynamic decreased levels were associated with a preferable prognosis in patients with PH. The results remind us of the role of NT-proBNP in PH and we reasonably postulate plasma betaine probably plays a similar role as a biomarker. Betaine changes could provide more information about the patient, and the lifestyle and diet of the patients included in the analysis are relatively

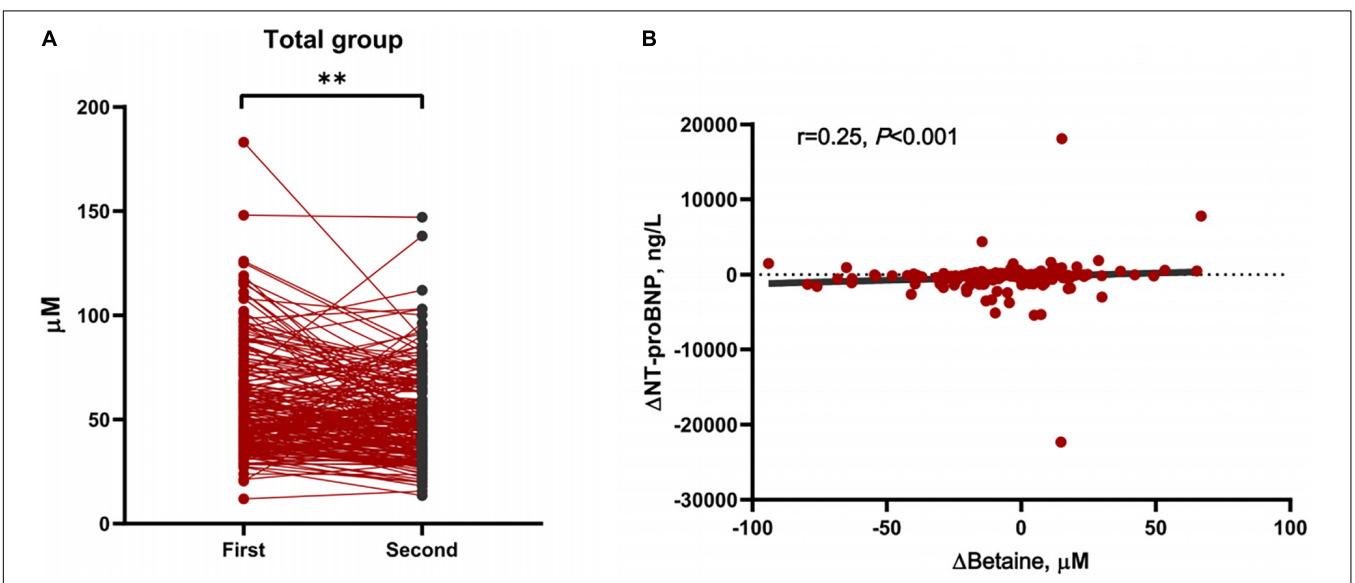

FIGURE 3 | Changes of betaine between first and second hospitalization and the association between Δbetaine and ΔN-terminal pro-brain natriuretic peptide (NT-proBNP). **(A)** Demonstration of plasma levels of betaine after further guideline-recommended treatment in all PH participants (n = 203). Data were compared using paired-samples t-tests. $^{**}p < 0.001$. **(B)** The positive association between Δbetaine and ΔNT-proBNP. Δbetaine and ΔNT-proBNP were defined as the value at the second admission visit minus the baseline value. Spearman's correlation (2-tailed) was used for analysis.

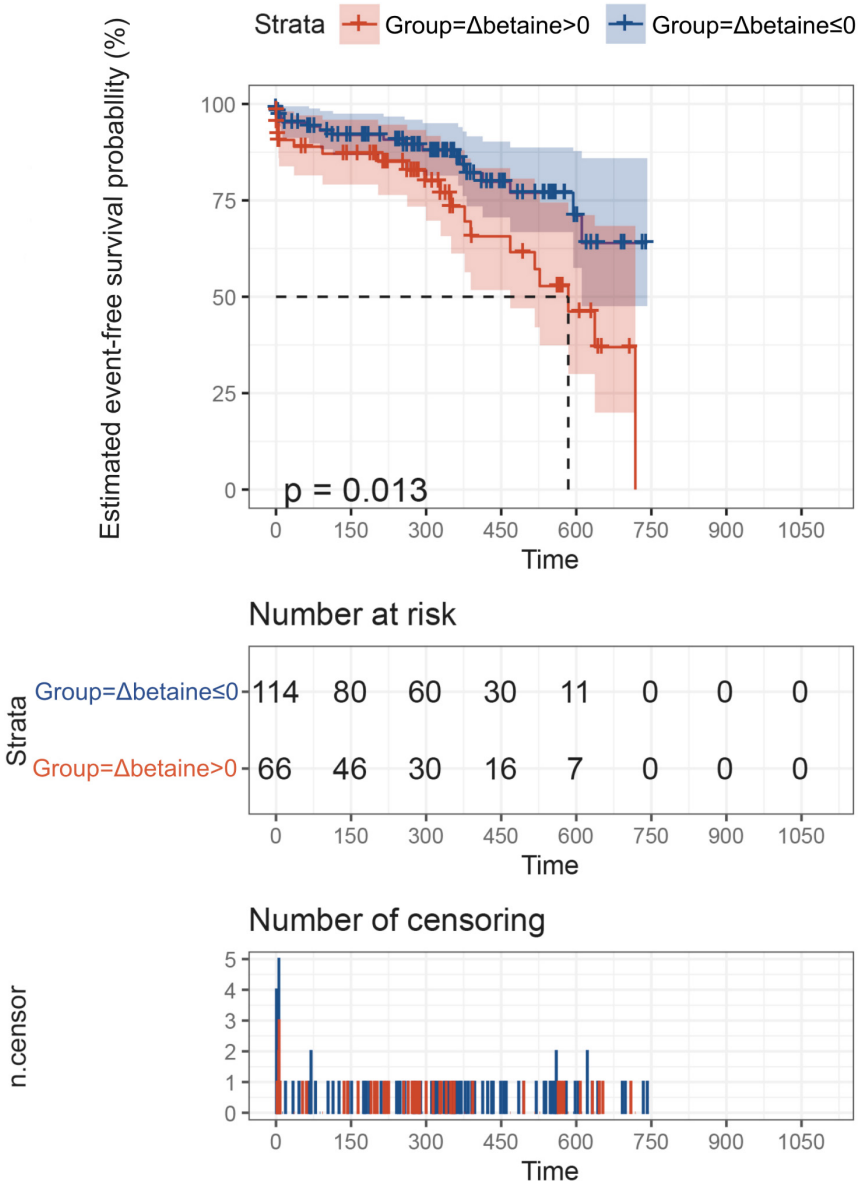

FIGURE 4 | ΔBetaine was associated with the prognosis of patients with PH. Patients were divided into two groups according to Δbetaine >0 or Δbetaine ≤0. Follow-up duration was defined from the second betaine measurement to the occurrence of outcomes or the end of follow-up. In this analysis, outcome events occurred in 23 patients between two measurement points, so these patients were excluded. Finally, a total of 180 patients with PH were included for exploration. Value of *p* calculated by the log-rank test.

consistent, which could largely reduce the effect of diet on betaine biosynthesis. In addition, Δbetaine was positively correlated with ΔNT-proBNP, indicating the dynamic change of betaine might reflect the disease condition and help for the management of PH.

The evidence showed that betaine enabled to protect internal organs, improve vascular risk factors, and enhance performance (8). The positive effects of betaine supplementation in treating hyper-homocystinurics (22, 23) and alcohol-induced hepatic steatosis (24) have also been elucidated. As a multifunctional agent, betaine possesses various physiological activities that include anti-oxidation, anti-inflammation, and anti-fibrosis (25–27). Notably, one study found that betaine exerted an anti-angiogenic activity ***in vivo*** and ***in vitro*** through the suppression of nuclear factor-κ B (NF-κ B) and Akt activation (28), another study showed betaine attenuated isoproterenol-induced acute myocardial ischemia *via* the regulation of signal transducer and activator of transcription 3 and apoptotic pathways (29). However, our study pointed out that high betaine levels were associated with poor clinical outcomes in patients with PH. Similarly, a meta-analysis also revealed that increased concentrations of betaine were correlated with the risk of major

adverse cardiovascular events (30). We postulated that the increase of betaine in plasma might be a consequence due to disease progression rather than a cause. Lever et al. (18) speculated that betaine might be leaking from tissues where it was accumulated as an osmolyte. The leakage was a part of pathology in disease indicating the metabolic failure in patients. More severe patients probably have more leakage resulting in higher metabolite concentrations in plasma. However, further studies are needed to dissect the underlying mechanisms involved in this association.

Our study is by far the largest of its kind to examine the relationship of plasma betaine with PH development and prognosis. The cohort included PH patients without connective tissue disease, immune disease, acute coronary syndromes, active infection, malignancy, congestive heart failure, or diabetes. As such, our findings are less likely confounded by other pre-existing disease conditions and medication usage. However, several potential concerns or limitations are worth mentioning. Firstly, we do not have information regarding the dietary patterns, which are significantly related to the betaine concentration detected in the blood (31). Similarly, no information was available regarding non-prescriptive dietary supplements that may have been utilized by patients at the time of the study. Moreover, as a single-center cohort, this research was conducted on Chinese populations, where dietary habits differ dramatically from that of western countries. As such, the prospective association between plasma betaine and PH remains uncertain in western populations. Regardless, we would like to emphasize that our study findings are preliminary and were just hypothesize generating. Since this work was designed as an observational study, the associations in the current study cannot be considered causal, all findings need to be further investigated by large-scale randomized trials. Our findings, if further confirmed, call for collaborative research effort in the next decade could lead to significant further progress and perhaps even a cure of PH.

CONCLUSION

The increase in plasma betaine was associated with PH severity and heralded a poor prognosis of patients with PH. Moreover, the dynamic rise of betaine level was also associated with adverse prognosis. Our study revealed the promising biomarker role of betaine and further explorations are calling for.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committees of Fuwai Hospital (Approval No: 2018-1063). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YY, JXu, JXue, and JZ contributed to the study design and interpretation of the results. YY, JXu, JXue, JZ, XL, BS, and BY contributed to the collection, analysis, or interpretation of data. YY and JXu prepared the manuscript. ZL, ZZ, QL, QZ, LZ, and CX critically revised the manuscript. All authors read and approved the final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.852009/full#supplementary-material

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