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Lowered Maternal and Paternal Plasma Concentrations of Choline are Associated with the Severity of Congenital Heart Defects in the Offspring

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Abstract

Background/Objectives: Congenital heart defects (CHD) are the most common structural birth defects that exhibit high heritability. Emerging evidence suggested that CHD are associated with disruptions in one-carbon metabolism. In a family-based trio design, we investigated whether maternal, paternal, and child plasma concentrations of choline, betaine, and folate were associated with CHD severity. **Subjects and Methods:** The study included 72 children with CHD, 69 mothers and 64 fathers of the children. CHD severity was classified according to the European network of population-based registries for the epidemiological surveillance of congenital anomalies (EUROCAT) system and the German PAN study (Prevalence of Congenital Heart Defects in Newborns). Plasma and urine concentrations of choline and betaine and plasma folate vitamers were quantified using ultra-performance liquid chromatography–tandem mass spectrometry. **Results:** The children [mean (SD) age 3.1 (3.2) years, 59.7% males] presented with varying CHD severities according to EUROCAT (62.5% severe and 37.5% mild) and PAN classifications (45.8% severe, 30.6% moderate and 23.6% mild). Plasma concentrations of choline were < 10 µmol/L in 38 (66.7%) of the mothers and 27 (62.8%) of the fathers who provided blood samples. Maternal plasma choline concentrations < 10 µmol/L were associated with having a child with severe CHD [adjusted odds ratio (aOR) 3.7; 95% confidence intervals (95%CI) = 1.1, 12.2 compared to mothers with choline concentrations ≥ 10 µmol/L]. Lowered paternal plasma choline concentrations were also associated with severe CHD (aOR 7.4; 95% CI = 1.7, 31.5). Plasma concentrations of choline in the children and those of betaine and folate vitamers in parents and children were not associated with CHD severity. **Conclusions:** Lower plasma concentrations of choline in the parents detectable several years after conception, were related to having a child with severe CHD compared with families of children with higher plasma choline. These findings support a potential role for maternal and paternal choline metabolism in modulating CHD severity. Etiological studies aiming at prevention of prevalent congenital anomalies should focus on maternal and paternal risk factors.

Keywords: betaine; children; choline; congenital heart defects; folate

1. Introduction

Congenital heart defects (CHDs) occur in approximately 1 in 100 live births and represent structural abnormalities of the heart walls, valves, or major blood vessels [1,2]. While many defects can be treated with surgical or medical interventions, even mild to moderate forms of CHD are linked to increased morbidity and mortality [2]. Maternal and paternal risk factors for CHDs remain

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insufficiently understood. However, observational studies suggest that maternal folic acid supplementation during pregnancy is associated with a reduced risk of CHDs in offspring [3–8]. Paternal risk factors and lifestyle habits are often overlooked, yet substantial evidence indicates that preconception factors in fathers can also significantly impact outcomes. Pregnancy and fetal development are characterized by a markedly increased demand for folate and other nutrients involved in one-carbon (C1)-metabolism.

Folate plays a central role in C1-metabolism by providing purine and pyrimidine nucleotides and methyl groups essential for DNA synthesis and methylation reactions [9,10]. The choline/betaine pathway also contributes substantially to cellular methylation, particularly under conditions of limited folate availability [11,12]. Choline is irreversibly oxidized to betaine (trimethylglycine), which donates a methyl group for the remethylation of homocysteine to methionine, producing dimethylglycine in the process [12]. Betaine-homocysteine methyltransferases (BHMT and BHMT2) catalyze the removal of homocysteine using methyl groups derived from choline/betaine (via BHMT) or folate-dependent pathways (BHMT2 utilizes S-adenosylmethionine) [13]. Choline also has distinct biological functions, including serving as a precursor for the neurotransmitter acetylcholine and as a structural component of cell-signaling molecules and membrane phospholipids.

Disturbances in choline metabolism have been associated with CHD in both clinical and experimental studies [14–18]. For instance, mothers of children with CHD exhibit marked dysregulation of choline-derived phospholipids in plasma at various stages of pregnancy and postpartum [14–16]. Altered biosynthesis of phosphatidylcholine and phosphatidylethanolamine has been detected in urine samples collected between 14 and 37 weeks of gestation from women who later delivered children with CHD [16]. Furthermore, low first-trimester serum concentrations of phosphatidylcholine derivatives—such as lysophosphatidylcholine and sphingomyelin—have been shown to predict CHD in offspring [19]. Metabolomic analyses in affected children have demonstrated higher plasma concentrations of betaine and choline compared with control children without CHD [20]. Collectively, independent studies showed consistent alterations in choline metabolism among pregnant women or children affected by CHD.

CHDs also exhibit high heritability estimates, ranging from 0.5 to 0.9 [21,22]. In line with this, maternal genetic variants affecting choline or folate metabolism have been linked to an increased risk of CHD in the children [23–25]. Moreover, our previous study of mother–child pairs showed that both children with CHD and their mothers had elevated concentrations of dimethylglycine, the demethylation product of betaine, compared with control pairs [26]. Together, these observations support the hypothesis that familial metabolic programming may be transmitted from parent to child and contribute to CHD etiology.

The present study tested the following hypotheses: (1) low plasma concentrations of choline or folate-related markers in the child are associated with greater CHD severity; and (2) low plasma concentrations of choline or folate-related markers in the parents are associated with increased CHD severity in the child. Additionally, we aimed to examine the relationship between plasma folate concentrations and both plasma and urinary levels of choline and betaine in affected children.

2. Materials and Methods

Subjects

This study of plasma choline and folate metabolites was conducted within the framework of the National Registers for Congenital Heart Diseases, a nationwide genetic research initiative designed to collect biospecimens from children with CHD and their parents (trio samples). The availability of trio samples enabled the assessment of choline and folate metabolites in both affected children and their parents.

Parents and their children with CHD were recruited at the Department of Pediatric Cardiology, Saarland University Hospital, Germany between January 2021 and May 2022. Consecutive children younger than 10 years of age with CHD of any severity, with or without associated syndromic

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conditions, were eligible for inclusion. Parents were enrolled if they agreed to participate. Exclusion criteria included conception by in vitro fertilization, the presence of congenital metabolic disorders in the child, and after surgical procedures or acute illnesses. Children were recruited during a scheduled hospital admission prior to any invasive surgical or catheter-based intervention. Blood samples were obtained before these procedures. A standardized questionnaire completed by the parents collected demographic data, obstetric history, and potential risk factors, including smoking, alcohol consumption, medication use, and vitamin supplementation. Additional information was obtained on the timing of CHD diagnosis, birth weight, and pregnancy-related complications. CHD severity was classified according to the EUROCAT (European network of population-based registries for the epidemiological surveillance of congenital anomalies) system [27] and the German PAN study (Prevalence of Congenital Heart Failure in the Newborns or Prävalenz angeborener Herzfehler bei Neugeborenen) [1].

Blood Collection and Laboratory Analyses

Blood samples were collected from the children into EDTA-K-containing tubes during a routine blood sampling as part of the biobanking study. Additionally, samples of spontaneous urine were collected from the children into dry tubes. Blood samples from the parents were collected into EDTA-K-containing tubes. The blood was centrifuged and the EDTA-plasma was collected into clean Eppendorf tubes. All samples were stored at -80°C until analyses of the biomarkers. The remaining EDTA-blood samples were forwarded to the biobank of the Competence Network for Congenital Heart Defects in Berlin, Germany.

Concentrations of plasma folate forms and plasma and urine choline and betaine were measured at the Central Laboratory of the Institute for Clinical Chemistry and Laboratory Medicine at Saarland University Hospital in Homburg, Germany. The concentrations of choline and betaine in plasma and urine were measured by using Ultra Performance Liquid Chromatography tandem mass spectrometry (LC-MS/MS) and isotope labelled d9-choline and d9-betaine chloride as internal standards as published elsewhere [28,29]. Urine samples were diluted 1:5 in water (20 µL urine and 80 µl water) before adding the acetonitrile-internal standards solution. EDTA-plasma (100 µl) or diluted urine (100 µl) samples were mixed with 300 µl of acetonitrile-containing the internal standards. After centrifugation at 10000 g for 10 min at ambient temperature, the supernatant was collected into a clean vial and measured on the UPLC-MS/MS system. Samples from the children and the parents were measured in the same batch to minimize analytical variations. Acquity Ultra Performance LC system coupled with a MicroMass Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA) was used for the measurements. Each sample preparation run included three quality control samples to capture the day-to-day variations, two levels of a solution of the pure compounds and in-house prepared pool EDTA plasma. The between-day coefficient of variations for the choline and betaine assay were < 7%.

The concentrations of creatinine in urine samples were measured by using the COBAS INTEGRA system (Roche Diagnostics, Mannheim). Concentrations of choline and betaine in urine were expressed as mmol/mol creatinine.

The individual folate forms were measured in EDTA-plasma using a validated method on UPLC-MS/MS as described elsewhere [28,30]. The method relies on isotope labelled folate forms and quantifies 5 different forms; (6S)-5-methyltetrahydrofolate (5-MTHF), (6S)- tetrahydrofolate, (6S)-5-formyltetrahydrofolate, (6R)-5,10-methelynetetrahydrofolate, and folic acid. Total folate was the sum of the individual forms. The none-methylfolate was the sum of all forms except 5-MTHF. The between-day CVs was 2% for 5-methyltetrahydrofolate and up to 11.2% for minor folate forms.

Statistical Analyses

The One-Sample Kolmogorov-Smirnov test with Lilliefors Significance Correction and Q-Q plots were used to study the data distribution. The concentrations of all biomarkers were not normally distributed (p values < 0.01), except those of plasma betaine in the children and the mothers and

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plasma 5-MTHF in the fathers. All variables (including those with normal distribution) were log-transformed. The distribution was improved after log-transformation and the log-transformed values were used for statistical tests that assume normal distribution of the data. The one-way analysis of variance (ANOVA) test was used to compare the log-transformed variables between more than two groups. Tamhane T2 test was used as a post-hoc test to correct for multiple comparisons when ANOVA test showed an overall p-value ≤ 0.05 and when the population variance was not homogenous ($p < 0.05$ from Levene statistics). The non-parametric Mann-Whitney test was used to compare continuous variables between two groups. Chi-square test was used to compare between groups differences in the distribution of categorical variables.

Although severe choline deficiency is not common, plasma choline concentrations below 10 μmol/L in adults may indicate low choline intake [31,32] compared to when the concentrations are higher than this limit. Logistic regression analyses were used to compute the odds ratio (OR) of the child to have a severe form of CHD when one of the parents had lowered plasma choline (< 10 μmol/L). Moreover, linear regression models were used to study the association between having a severe form of CHD and child or parents' concentrations of choline, betaine, and folate that were used as continuous variables to investigate whether associations may exist over the whole concentrations range of these biomarkers.

The regression models that tested child exposures (i.e., choline, betaine or folate) were adjusted for the age of the child. When studying maternal exposures, the regression analyses were adjusted for child age, mother age and smoking habit in the mother (yes or no). Alternatively, the regression models were adjusted for child age and father age when studying paternal exposures. We decided not to adjust for additional self-reported variables, especially those related to the index pregnancy because of potential reporting bias. Each regression model included only one biomarker at a time. For example, the model concerned with choline as the exposure of interest did not adjusted for folate or betaine. For the primary hypothesis testing of the association between plasma biomarkers and CHD severity, the EUROCAT system was used to classify CHD severity. Sensitivity analyses were performed to study the associations of the biomarkers with CHD severity according to the PAN study.

Multivariate linear regression analysis was used to explore determinants of urinary betaine concentrations in the child (outcome variable). We investigated whether child age, plasma folate, plasma choline, plasma betaine or severity of the CHD lesion were significant predictors of urinary betaine. Continuous variables in this model were entered as log-transformed values.

We used version 31 of IBM® SPSS® Statistics package (SPSS Inc., Chicago, IL, USA). P-values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Characteristics of the Study Population

Seventy-five children fulfilled the inclusion criteria and none of the exclusion criteria. Parents' consent was available from 72 children. Among the patents, n = 69 mothers and n = 64 fathers agreed to fill the study questionnaire. Blood samples were available from 59 children, 57 mothers and 43 fathers; with 30 families where the three of them contributing to the study with blood samples in the same time.

The mean (SD) age of the children was 3.1 (3.2) years (59.7% were boys). According to the EUROCAT classification, 45 children (62.5%) were diagnosed with moderate CHD lesions. Based on the PAN study classification, 22 children (30.5%) had moderate CHD and 33 (45.8%) had severe CHD (Table 1). The mean (SD) maternal age was 32.1 (4.3) years at the birth of the index child and 35.0 (4.4) years at study recruitment. Seventeen mothers (24.6%) reported current smoking. The mean (SD) paternal age at recruitment was 37.8 (6.5) years.

Table 2 summarizes the distribution of specific CHD diagnoses and their categorization according to the EUROCAT and PAN classification systems.

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Table 1. Main characteristics of 72 children with CHD and their parents. .

	All
Age, years (n = 72)	3.1 (3.2)
Boys, n (%)	43 (59.7%)
Height, cm (n = 66)	84 (27)
Weight, kg (n = 69)	12 (8)
Severity of CHD, n (%)	
Mild	17 (23.6%)
Moderate	22 (30.6%)
Severe	33 (45.8%)
Severity of CHD (EUROCAT classification), n (%)	
Mild	27 (37.5%)
Severe	45 (62.5%)
Syndrome, n (%)	14 (19.4%)
Mother age at birth of the index child, years (n = 66)	32.1 (4.3)
Mother age, years (n = 69)	35.0 (4.4)
Mother BMI, kg/m^2 (n = 65)	26.9 (6.8)
Current smoker, n (%)	17 (24.6%)
Parity, n (%)	
1	26 (37.7%)
2	25 (36.2%)
≥ 3	18 (26.1%)
Father age, years (n = 64)	37.8 (6.5)
Father BMI, kg/m^2 (n = 57)	27.4 (3.9)

Data are shown as mean (SD), unless otherwise specified. BMI, body Maas Index; CHD, congenital heart disease; EUROCAT, European network of population-based registries for the epidemiological surveillance of congenital anomalies.

Table 2. Diagnosis of CHD and severity according to EUROCAT and the PAN Study. .

		CHD severity	
CHD Type	**N cases**	**EUROCAT classification [27]**	**PAN study classification [1]**
Atrial septal defect (ASD II), Sinus venosus atrial septal defect (SVASD)	11	mild	mild
Ventricular septal defect (VSD)	11	mild	mild (when small or muscular VSD); moderate (for all other VSD)
Single ventricle	8	severe	severe
Complex CHD, not classifiable	6	severe	severe
Tetralogy of Fallot (TOF)	6	severe	severe
Atrioventricular septal defect (AVSD)	5	severe	moderate
Transposition of the great arteries (TGA)	5	severe	severe
Coarctation of the aorta (CoA)	6	severe	moderate
Aortic stenosis (AS)	3	severe	moderate
Anomalous pulmonary venous return (APVR)	2	severe	moderate (partial anomalous pulmonary venous return, PAPVC); severe (total anomalous pulmonary venous return, TAPVC)
Pulmonary atresia (PA)	2	severe	severe
Tricuspid atresia (TA)	2	severe	severe
Patent ductus arteriosus (PDA)	3	mild	mild
Truncus arteriosus communis (TAC)	1	severe	severe
Hypoplastic left heart syndrome (HLHS)	1	severe	severe

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3.2. Plasma Concentrations of Choline, Betaine and Folate in Relation to CHD Severity

Plasma concentrations of choline and folate biomarkers in the children did not differ according to CHD severity (Table 3). Plasma concentrations of choline, betaine, and folate did not differ between boys and girls (data not shown). Urinary betaine concentrations were significantly higher in children with moderate and severe CHD compared with those with mild CHD, based on the PAN classifications (Table S1).

Maternal plasma concentrations of choline, betaine, and folate did not significantly differ according to disease severity in the child, although choline concentrations tended to be lower in mothers of children with severe CHD (Table 3, Table S1). Paternal plasma choline concentrations were significantly lower in fathers of children with severe CHD compared with fathers of children with mild or moderate CHD, according to EUROCAT and PAN classifications (Table 3, Table S1). A higher proportion of mothers (77% vs. 50%, p = 0.046 Chi-Square test) and fathers (81% vs. 35%, p = 0.004 Chi-Square test) had low plasma choline concentrations (< 10 µmol/L) in the group with severe CHD compared to that with mild CHD (Table 3).

Table 3. Plasma concentrations of choline and folate markers in cases with congenital heart disease (CHD) and their parents in total and according to EUROCAT classification of CHD severity. .

	All	Mild CHD	Severe CHD	P[1]
Child				
Age, weeks	3.1 (3.2) 2.1 [4.0]	2.7 (2.5) 1.7 [3.6]	3.3 (3.5) 3.4 [4.2]	0.972
Plasma choline, µmol/L (n = 56)	14.0 (10.0) 11.4 [8.9]	16.1 (12.8) 14.1 [9.4]	13.0 (8.4) 12.3 [8.5]	0.261
Plasma choline < 10 µmol/L, n/total	20/56	4/18	16/38	0.233[2]
Plasma betaine, µmol/L (n = 57)	52.7 (17.5) 51.8 [27.9]	53.4 (16.5) 54.9 [21.2]	52.4 (18.2) 50.3 [31.1]	0.520
Urinary choline, mmol/mol creatinine (n = 57)	1.54 (2.44) 0.7 [1.2]	2.0 (3.4) 0.8 [1.8]	1.2 (1.6) 0.7 [1.0]	0.589
Urinary betaine, mmol/mol creatinine (n = 58)	68.1 (100.1) 9.0 [104.2]	72.0 (123.9) 7.3 [110.9]	65.7 (84.3) 30.9 [105.9]	0.423
Plasma total folate (sum of folate forms), nmol/L (n = 59)	12.6 (11.3) 10.2 [12.5]	12.5 (13.5) 6.7 [12.1]	12.6 (10.1) 11.1 [12.6]	0.442
Plasma 5-MTHF, nmol/L (n = 59)	10.8 (9.4) 7.2 [11.4]	11.1 (11.7) 5.7 [11.3]	10.6 (8.1) 8.8 [11.8]	0.608
Plasma non-methyl-THF, nmol/L (n = 59)	1.8 (5.6) 0.5 [1.0]	1.4 (2.6) 0.8 [1.3]	2.0 (6.7) 0.4 [0.7]	0.586
Mother markers				
Plasma choline, µmol/L (n = 57)	9.5 (5.1) 7.7 [5.5]	10.5 (4.7) 9.9 [7.9]	8.8 (5.2) 7.4 [3.9]	0.096
Plasma choline < 10 µmol/L, n/total	38/57	**11/22**	**27/35**	**0.046[2]**
Plasma betaine, µmol/L (n = 57)	36.0 (12.6) 34.8 [17.0]	36.4 (13.2) 37.4 [15.4]	35.8 (12.4) 33.3 [15.6]	0.682
Plasma total folate (sum of folate forms), nmol/L (n = 65)	7.4 (7.2) 4.9 [5.5]	5.1 (3.9) 3.7 [3.1]	8.8 (8.3) 5.3 [10.8]	0.126
Plasma 5-MTHF, nmol/L (n = 65)	6.2 (5.8) 4.2 [4.2]	4.3 (3.8) 3.1 [3.0]	7.3 (6.5) 4.9 [8.8]	0.087
Plasma non-methyl-THF, nmol/L (n = 65)	1.2 (3.2) 0.3 [0.7]	0.7 (1.3) 0.2 [0.9]	1.6 (3.9) 0.3 [0.7]	0.780
Father markers				
Plasma choline, µmol/L (n = 43)	10.3 (5.4) 8.1 [9.2]	**12.2 (4.5)** **10.7 [8.5]**	**9.1 (5.7)** **7.1 [4.0]**	**0.008**
Plasma choline < 10 µmol/L, n/total	27/43	**6/17**	**21/26**	**0.004[2]**
Plasma betaine, µmol/L (n = 44)	41.5 (10.8) 38.1 [10.2]	40.5 (9.4) 37.1 [9.3]	42.2 (11.8) 39.7 [12.4]	0.772
Plasma total folate (sum of folate forms), nmol/L (n = 50)	3.8 (3.0) 3.4 [2.0]	2.9 (1.4) 2.5 [2.2]	4.4 (3.5) 3.6 [2.4]	0.050

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Plasma 5-MTHF, nmol/L (n = 50)	3.1 (1.7) 2.8 [2.0]	2.5 (1.2) 2.4 [2.0]	3.5 (1.9) 3.4 [2.1]	0.069
Plasma non-methyl-THF, nmol/L (n = 50)	0.7 (2.6) 0.1 [0.4]	0.3 (0.3) 0.3 [0.5]	0.9 (3.3) 0.1 [0.3]	0.161

Results are shown as mean (SD) and median [IQR] unless otherwise specified. [1] P values from the Mann-Whitney U Test. [2] Chi-square test was used to compare categorical variables between the severity groups. Results in bold are statistically significant. CHD, congenital heart disease; EUROCAT, European network of population-based registries for the epidemiological surveillance of congenital anomalies; 5-MTHF, 5-methyltetrahydrofolate.

We further examined the associations between parental plasma biomarkers and CHD severity in the offspring (Table 4, Table S2). Maternal plasma choline concentrations below 10 μmol/L were associated with up to a 3.7-fold higher risk of severe CHD in the child compared with concentrations ≥10 μmol/L (adjusted OR [95% CI]: 3.7 [1.1, 12.2] for severe CHD according to EUROCAT; 3.3 [1.0, 11.0] according to the PAN classification). Similarly, low paternal plasma choline concentrations were associated with an increased risk of severe CHD in the child (adjusted OR [95% CI]: 7.4 [1.7, 31.5] for severe CHD according to EUROCAT (Table 4) and 4.0 [0.9, 17.4] according to the PAN classification (Table S2). When analyzed as a continuous variable in linear regression models, parental plasma choline concentrations remained significantly associated with CHD severity in the child, suggesting that the association is present across the range of plasma choline.

Plasma concentrations of choline were available from 37 mother-father pairs. In 19 families, both parents had low plasma choline concentrations; among these, 16 children had severe CHD (EUROCAT classification). In contrast, among the 18 families in which only one parent had low choline concentrations or both parents had normal levels, only 6 children had severe CHD.

No significant associations were observed between plasma betaine or folate concentrations—either in children or in parents—and CHD severity.

Table 4. Associations of child, maternal and paternal biomarker concentrations with severity of CHD in the child according to EUROCAT classification.

	n mild/ severe CHD	**Crude OR (95%CI)**	**Adjusted OR (95%CI) [1,2]**
Mother p-choline ≥ 10 μmol/L (n = 19 of 57 mothers)	11/8	OR = 1	
Mother p-choline < 10 μmol/L (n = 38 of 57 mothers)	11/27	**3.7 (1.2, 11.9)**	**3.7 (1.1, 12.2)**
Father p-choline ≥ 10 μmol/L (n = 16 of 43 fathers)	11/5	OR = 1	
Father p-choline < 10 μmol/L (n = 27 of 43 fathers)	6/21	**7.7 (1.9, 31.0)**	**7.4 (1.7, 31.5)**
Mother and/or father's p-choline ≥ 10 μmol/L , n = 18	12/6	OR = 1	
Both mother and father's p-choline < 10 μmol/L, n = 19	3/16	**10.7 (2.2, 51.5)**	**11.1 (2.1, 59.3)**
Exposures as log-transformed continuous variables		**β-coefficient (95%CI)**	**Adjusted β-coefficient (95%CI)[1,2]**
Mother p-choline		-0.53 (-1.12, 0.07)	**-0.61 (-1.20, -0.02)[3]**
Mother p-betaine		0.04 (-0.67, 0.75)	-0.06 (-0.77, 0.65)
Mother p-folate		0.23 (-0.04, 0.62)	0.32 (-0.02, 0.65)
Father p-choline		**-0.85 (-0.49, -0.20)**	**-0.87 (-1.58, -0.16)[3]**
Father p-betaine		0.31 (-1.13, 1.76)	0.21 (-1.23, 1.65)
Father p-folate		0.54 (0.07, 1.01)	0.50 (0.01, 0.99)
Child p-choline		-0.30 (-0.77, 0.17)	-0.317 (-0.80, 0.17)
Child p-betaine		-0.06 (-0.85, 0.73)	-0.04 (-0.88, 0.80)
Child p-folate		0.11 (-0.20, 0.41)	0.16 (-0.17, 0.49)

[1] logistic and linear regression models for maternal exposures are adjusted for age of the mother, age of the child and maternal smoking (yes or no). [2] logistic and linear regression models for paternal exposures are adjusted for age of the father and age of the child. [3] The linear regressions show an inverse association between choline concentrations and CHD severity. Thus lower choline concentrations were associated with severe CHD (CHD

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forms were coded as 1 (for mild CHD) and 2 (for severe CHD) according to EUROCAT classification. OR and (95%CIs) were computed by running logistic regression analyses with the severity of the CHD entered in the model as an outcome variable (1 = mild; 2 = severe). The exposure variable (plasma choline in the mother or the father < 10 μmol/L vs. ≥ 10 μmol/L) and the model-specific covariates were entered as independent variables. The beta coefficient (β) and 95%CIs were computed from a Generalized Linear Model (GLM) using the severity of the CHD as an outcome variable. One exposure variable (e.g., log-transformed plasma choline in the mother or the father) alone or with the covariates were entered as predictor variables. Results in bold are statistically significant. CHD, congenital heart disease; EUROCAT, European network of population-based registries for the epidemiological surveillance of congenital anomalies; p-choline (betaine or folate), plasma choline (betaine or folate).

3.3. Interaction Between Choline and Folate Markers in the Children

Plasma choline and urinary metabolite concentrations in the children were analyzed according to tertiles of plasma folate. Higher plasma folate concentrations were associated with higher plasma choline and increased urinary betaine levels (Table S3). In contrast, plasma betaine concentrations were not significantly associated with plasma folate levels (Table S3).

In multivariable linear regression analyses, elevated urinary betaine concentrations were independently predicted by greater CHD severity, higher plasma folate, and higher plasma choline concentrations. The child's age was not a significant determinant of urinary betaine levels (Table S4).

4. Discussion

In this study, we examined circulating biomarkers of choline and folate in blood samples from children with CHD and their parents in relation to CHD severity. We found that lower parental plasma choline concentrations were associated with a substantially increased risk of severe CHD in the offspring. Notably, when both parents had reduced plasma choline concentrations (<10 μmol/L), the likelihood of severe CHD appeared to be even higher compared with families in which choline levels were normal in both parents or reduced in only one parent. The findings suggest additive effects of maternal and paternal risk factors and a dose–response relationship between parental plasma choline concentrations and CHD severity. In contrast, plasma choline in the children and plasma betaine and folate in either children or parents were not associated with CHD severity.

In this study, parents of children with severe CHD exhibited alterations in the choline pathway that were detectable several years after conception, compared with parents of children with mild or moderate CHD. The lack of associations between child choline levels and CHD severity could be due to generally high plasma concentrations of choline in the children until adulthood [33]. Previous studies have reported associations between maternal genetic polymorphisms in the BHMT gene and CHD risk in offspring, supporting the concept of a familial pattern of disturbed choline metabolism [24,25]. Our findings further raise the possibility that paternal metabolic or genetic factors may also contribute to CHD susceptibility. Together, these observations suggest that inherited or shared metabolic characteristics—potentially influenced by both genetics and long-term nutritional status—may play a role in CHD severity.

Choline is an essential nutrient involved in liver function, lipid metabolism, and homocysteine remethylation. The European Food Safety Authority (EFSA) recommends a daily intake of 480 mg for pregnant women and 400 mg for non-pregnant women and men [34], while the U.S. National Academy of Medicine recommends 550 mg/day for adults [34], with slightly lower requirements for non-pregnant pre-menopausal women due to estrogen-stimulated endogenous choline synthesis [35]. However, epidemiological data indicate that average dietary choline intake is typically 100–200 mg/day below these recommendations [36,37]. A recent observational study from China reported that higher choline intake during pregnancy was associated with a lower risk of CHD [38]. In that study, median total choline intake (190 mg/day in the cases and 247 mg/day in the controls) was relatively low in both cases and controls, and each 50 mg/day increase in choline intake was associated with an estimated 15% reduction in CHD risk [38]. Currently, most prenatal supplements

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do not contain choline, and there are no formal recommendations regarding paternal choline supplementation during preconception. Our findings, together with previous genetic and nutritional studies [24,25], support further investigation into whether optimizing parental choline status before and during pregnancy could reduce the risk of severe CHD. Moreover, whether increasing choline intake from foods or food supplements can influence cardiac development, remains to be clarified. Genetic testing of prospective parents or those who already had a child with CHD is not cost effective, while increasing choline intake on a population level may be the better approach. In addition, measuring homocysteine after oral methionine load (e.g., 5.6g to 7.5g methionine for a 75 kg person) can detect disorders in the BHMT pathway not detected by measuring fasting plasma homocysteine [39–41]. Post-methionine load homocysteine is also sensitive to insufficient choline intake [42–44] and it holds potential as a screening test that enables better targeting of C1-metabolism in high risk couples.

An additional novel finding of this study was the association between higher urinary betaine excretion and higher plasma folate concentrations in children with CHD. To our knowledge, this relationship has not been previously described. One possible explanation is a shift in homocysteine remethylation pathways depending on folate availability. Under conditions of high folate status, increased activity of folate-dependent remethylation pathways via methionine synthase may spare dietary choline and betaine, resulting in greater urinary excretion of betaine. This hypothesis requires further mechanistic investigation.

Although the present study cannot establish causality due to its observational design, experimental evidence supports a role for sufficient choline intake in normal cardiac development. Animal models have demonstrated that maternal choline deficiency (mice fed with 1/8 choline for 6 weeks before matting) [17] can lead to structural heart defects, including ventricular septal defects, and may impair normal cardiac development compared to when animals were fed a standard diet [17]. In mouse models with impaired folate metabolism due to knockout of the methylenetetrahydrofolate reductase (MTHFR) gene, adequate maternal choline intake through the diet has been shown to partially rescue cardiac development [17]. Furthermore, experimental studies suggest cardioprotective effects of choline derivatives and betaine under conditions of prenatal stress or injury [45]. Also betaine has been shown to prevent the effect of prenatal exposure to ethanol on the heart of the offspring [18]. In betaine-supplemented embryos, great vessel diameters, interventricular septum thickness, and atrioventricular leaflet volumes were rather similar to those in the control embryos [18], thus showing analogy to a possible role of choline in normal heart development. Collectively, these findings support a biologically plausible role for choline in embryonic heart development, particularly when folate metabolism is compromised.

This study has several limitations, including the relatively small sample size and the use of an observational design within an existing cohort. The limited sample size may have reduced our ability to detect weaker associations. Nonetheless, metabolic studies provide important complementary evidence to genetic research and may help elucidate underlying mechanisms.

5. Conclusions

In conclusion, low parental plasma choline concentrations were associated with a several-fold increased risk of severe CHD in the offspring, with the highest risk observed when both parents had choline levels below 10 μmol/L. In contrast, the child's own plasma choline concentrations were not related to lesion severity. Future studies integrating metabolic profiling with genetic analyses may help identify modifiable risk factors and clarify the potential role of insufficient parental choline status in development of severe CHD. Etiological studies aiming at prevention of prevalent congenital anomalies should focus on maternal and paternal risk factors.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, **Table S1**: Plasma concentrations of choline and folate markers in children with congenital heart disease (CHD) and their parents. The data are shown for the whole group and by severity of

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CHD according to the PAN study classification. **Table S2**: Associations of maternal and paternal biomarker concentrations with severity of CHD in the child classified according to the PAN study. **Table S3**: Plasma and urinary betaine and choline concentrations in children with CHD according to tertiles of plasma folate in the children; **Table S4**: Multivariate linear regression analyses to predict urinary concentrations of betaine in children with congenital heart disease (as an outcome variable).

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Institutional Review Board Statement: The study protocol was reviewed and approved by the local ethics committee of Saarland (Ärztekammer des Saarlandes; approval number: 54/20). The study was conducted in accordance with the ethical principles of the World Medical Association (Declaration of Helsinki) and the International Council for Harmonisation Good Clinical Practice (ICH-GCP) guidelines.

Informed Consent Statement: Written informed consent was obtained from the parents and on behalf of their children.

Data Availability Statement: Data can be provided in aggregate form to scientists upon reasonable request to the corresponding author.

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Abbreviations

The following abbreviations are used in this manuscript: BHMT, betaine-homocysteine methyltransferase; CHD, congenital heart defects; EFSA, European Food Safety Authority; EUROCAT, European network of population-based registries for the epidemiological surveillance of congenital anomalies; 5-MTHF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reeducate; PAN, Praevalenz angeborener Herzfehler bei Neugeborenen.

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