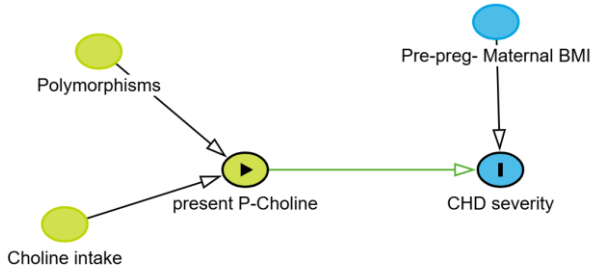
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Lowered maternal and paternal plasma concentrations of choline are associated with the severity of congenital heart defects in the offspring (Obeid et al.,)

Reply to comments of Reviewer 1

Comments	Authors reply
General assessment: The obtained results may have potential practical implications for guiding dietary and supplementation recommendations. However, some methodological and interpretative issues require clarification before publication.	We thank the reviewer for the constructive comments. We followed the suggestions and improved the paper.
Introduction: It provides basic information on the metabolic effects of paternal factors before conception, and this aspect should be expanded upon to justify the research.	Good point, thank you. *Changes in the paper: we added more information about paternal factors in the second paragraph of the introduction.*
Methods: There are several points that require clarification in this section. The main limitation of the study stems from the timing of biomarker measurement, which raises problems with the interpretation of the results. Biomarkers were measured on average 3.1 years after the child's birth, a time distant from the periconceptional period. This raises the question of whether current choline concentrations truly reflect periconceptional status and whether the observed association may be due to dietary patterns rather than a direct effect on embryogenesis.	We fully agree with the reviewer that choline concentrations measured at the age of 3 years cannot be considered representative for pre-conceptional choline. However, as mentioned in the introduction, alterations in choline metabolism have been reported in children or children and their mothers at a similar age beyond the etiology time window [1;2]. Given the study design, we cannot claim that our results are causally related to CHD during embryogenesis. We collected data on frequency of consuming key foods (such as meat, fish, dairy products, etc) per week. We found no differences in dietary pattern of the parents according to severity of the child CHD. Although we did not estimate choline intake from the data (due to lack of food tables), difference in choline intake of the parents according to severity of CHD in the child may not be a reasonable explanation for the results. *Changes in the paper: we revised the introduction and the discussion to reflect on this important aspect. Results section: "Five mothers and 2 fathers reported adhering to a vegetarian diet and 17 mothers and 6 fathers were low meat eaters. No association was found between the frequency of consuming meat, fish, and dairy products (per week) in the parents and plasma choline or the severity of CHD in the child".* 1. Alsayed R, et al (2013) Elevated dimethylglycine in blood of children with congenital heart defects and their mothers. Metabolism 62:1074-1080.

	2. Yu M, et al (2018) Discovery and Validation of Potential Serum Biomarkers for Pediatric Patients with Congenital Heart Diseases by Metabolomics. J Proteome Res 17:3517-3525.
The methodology lacks information regarding the diets of parents and children – were such questions asked in the questionnaire?	The questionnaire included questions on frequency of consuming food categories (meat, fish, dairy products, etc). We found no evidence that the frequency of consumption of any of the captured foods is related to plasma choline or severity of CHD.
Furthermore, the issue of vitamin supplementation during pregnancy requires clarification – whether it was administered and, if so, with which preparations.	We asked the mothers about using supplemental folic acid during pregnancy. The questionnaire did not inquire about preparation (i.e., the dose), because the delivery is a while ago and mothers may not remember the name of the prenatal products. Most prenatal supplements contain 400 µg and some may contain 800 µg folic acid/or folate. No products in Germany contained choline at the time when the women were pregnant. *Changes in the paper: We added the information about supplements use in pregnancy (Supplemental table S1). We think that the retrospective and self-reported information may not be accurate. Therefore, we did not extend elaboration on this point.*
Another concern is the study's inclusion criteria. The authors acknowledge that the study included children with CHD with and without associated syndromes. It would be helpful to specify which associated syndromes are being addressed, as, for example, genetic syndromes can affect one-carbon metabolism.	We appreciate the point raised by the reviewer. In total the study included 14 syndromic cases (8 Down syndrome; n = 1 Williams-Beuren-Syndrome (Chr7), n = 1 Turner syndrome, n = 1 microdillion 22q11, n = 1 Ivemark's syndrome, n = 1 Heterotaxy syndrome, n = 1 CHARGE-Syndrom). *Changes in the article: We presented the syndromic cases in Table 1. Moreover, we run a sensitivity analysis after excluding 14 children with syndromes. The results are shown now in Table S4. Fathers plasma choline (p = 0.003) and mother plasma choline (not significant) remained lower in severe CHD compared to mild CHD. Maternal choline was not statistically significant, possibly due to lower sample size. Child choline remained not different between severe and mild cases.*
It is also not fully clear whether BMI, socioeconomic status, supplementation, dietary choline intake, or genetic factors (e.g., MTHFR, BHMT) were controlled for. Considering these variables when studying one-carbon metabolism is particularly important.	We did not control for the mentioned factors. Adjustments were made for variables that may influence the outcome or the exposure and outcome at the same time (e.g., age of the mother, age of the child and maternal smoking). Data on dietary choline intake are not available to us. Moreover, dietary choline is a precursor of the exposure and should therefore not be adjusted for (one could alternatively ask the alternative on whether dietary choline intake is associated with the severity of CHD) (please see the corresponding figure for the DAG).

	Genetic factors and use of supplements may influence plasma choline (the exposure) and are therefore not confounders (for genetic factors, one could alternatively run a mendelian randomization study). Pre-pregnancy maternal BMI may influence choline intake or status and the severity of CHD, and may be a confounder. However, maternal BMI during pregnancy was not known to us. We think that we should not adjust for the mother present BMI, because present BMI may not be related to pre-pregnancy BMI.  *Changes in the paper: none.*
Another issue is that the threshold of < 10 µmol/L for choline is not a universally recognized clinical cut-off for deficiency - it is rather an interpretative value and this aspect needs to be emphasized in the paper.	We fully agree with the reviewer. The cutoff 10 µM is not based on strong evidence, neither aims to draw conclusions on choline sufficiency. For this reason, we also run a linear regression analyses to see whether the associations exist over the whole range of plasma choline. *Changes in the paper: we emphasized this point in the results: "The cut off for plasma choline (i.e., 10 µmol/L) is not based on strong evidence; neither aims to draw conclusions on choline sufficiency. Therefore, we analyzed the association between CHD severity and plasma choline as a continuous variable in linear regression models".*
The number of fathers with available data was only 43–44 in the biomarker analyses, which may be important for the obtained results and requires the possibility of a Type I error discussion.	The number of fathers accompanying their children and accepting to take part in the study is lower than the number of the mothers. Incorrect inference due to Type 1 error (or finding an association that does not exist) may occur due small sample size. However, on the one hand the results of plasma choline and CHD severity in the mothers and fathers are consistent. On the other hand, and independent from the statistical significance, there is a dose-response relationship which is unlikely to be due to chance. *Changes in the paper: we briefly addressed this point in the study limitations.*
There is no information as to why samples were missing.	*Changes in the paper:* *"Blood samples were missing from 17 children due to either technical difficulties during blood collection or a limited*

	amount of blood and the need to prioritize the routine analyses over the study. Blood samples were missing from 14 of the 64 fathers and 4 of the 69 mothers who agreed to participate. The reason for missing parent's samples was either that they were not accompanying the child or not willing to provide a blood sample to the study".
There is no information on whether collinearity in the models was checked and whether interactions were tested, which could increase the value of the work.	Colinearity in linear regression model was tested. *Changes in the paper: "Multicolinearity in linear regression analysis was studied by calculating the Variance Inflation Factor (VIF) that was below 1.3 for all variables in this model, thus indicating no colinearity".*
Discussion: The discussion should be more balanced regarding the obtained results, and the paragraph on Limitations should emphasize more the issues related to the number of studies, the time of analysis and the potential influence of lifestyle and diet on the results.	*Changes in the paper: We revised the discussion and considered the three aspects suggested by the reviewers in the study limitations.*
References: References: please ensure full and properly formatted references with DOI. The completeness of the references requires checking and correction. The paper [12] from 2025 in Nutrients may be "ahead of print" or potentially an article from the same group - it requires checking whether it was actually published (risk of self-plagiarism extension of the concept). Citations 38 need to be verified – the full data in the references is missing.	Thank you, we checked the references and added the DOIs. We had 4 self-citations, 3 of them are needed due to methods and previous studies. Reference 12 was a self-citation, now removed and replaced by another reference from other researchers.
Comments on the Quality of English Language: There is a mistake in the abbreviations section: "methylenetetrahydrofolate reeducate" instead of "reductase"	Done
In the text: Prevalence of Congenital Heart Failure in Newborns. This is an error – PAN refers to: Prevalence of Congenital Heart Defects in Newborns. Reference [1] is correct.	Done