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Higher Incidence of Common Polymorphisms in the Genes of Folate and Methionine Cycles in Children With Orofacial Clefs and Congenital Heart Defects Compared to their Unaffected Siblings

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ABSTRACT

Background: Uninterrupted folate metabolism plays a vital role in embryonic development, ensuring a supply of one-carbon-activated folate cofactors for essential processes. Folate deficiency has been implicated in the development of orofacial clefts (OFC) and congenital heart disease (CHD). Although both malformations have been extensively studied in lieu of folate deficiency, the results of corresponding studies are ambiguous due to the interplay of maternal and fetal genomes controlling folate metabolism in the developing fetus.

Methods: We used the innovative study design to compare affected and unaffected siblings from the same mother, thus minimizing the effect of the maternal genome. Thus, it might be possible to identify genetic markers of congenital malformations that pertain exclusively to the child. This study compared demographic and environmental factors between OFC or CHD-affected and unaffected pregnancies as well as the presence of polymorphisms in genes of folate metabolism between OFC or CHD-affected and unaffected siblings.

Results: Only the maternal fever in the first trimester was a risk factor for OFC, whereas the maternal advanced age, medication administration, and common polymorphism in the *FPGS* gene increased the risk of CHD formation. Both OFC and CHD formation were associated with a higher number of variant loci in genes of folate–methionine cycles.

Conclusions: Both OFC and CHD formation were associated with a higher number of mutated loci in genes of folate–methionine cycles, indicating polygenic and possibly multifactorial inheritance.

1 | Introduction

Orofacial clefts (OFC) and congenital heart defects (CHD) are among the most common congenital malformations in humans, with a worldwide frequency of 1 in 700 (Mossey et al. 2009) and 1 in 100 (van der Linde et al. 2011) births, respectively. Both congenital malformations represent significant health burden for the patients and their families, as well as for societies and

health systems. Therefore, the identification of demographical, environmental, and genetic risk factors for the development of OFC and CHD is of the utmost importance for their prevention and therapy development.

Although both OFC and CHD have been extensively studied, their etiology remains unclear, indicating complex multifactorial inheritance. Several demographic and environmental risk factors have been detected so far for OFC (maternal vitamin and folate supplement use during pregnancy, maternal smoking, maternal hyperhomocysteinemia, use of certain drugs, and the incidence of fever during pregnancy) (Mossey et al. 2009) and CHD (maternal folic acid deficiency, maternal diabetes, fever in first trimester, maternal chronic disease, advanced maternal age, and maternal drug exposure) (Abqari et al. 2016). The most prominent genetic regions linked to OFC are situated on chromosomes 1, 2, 4, 6, 14, 17, and 19 and contain genes from various categories, including growth factors, transcription factors, xenobiotic metabolism, immune response, and folate metabolism (Mossey et al. 2009). Genetic loci associated with isolated CHD are located on chromosomes 1, 3–9, 11, 14, 15, 17, 18, 20, and 22 and contain mostly genes coding for transcription factors, transcription coactivators, growth factors and extracellular matrix structural constituents (Azhar and Ware 2016).

Folates have been heavily implicated in the normal development of the central neural system. The lack of active forms of folate (Kancherla 2023) and mutations in the genes of folate/methionine cycles (Almekkawi et al. 2022) has been identified as a major risk factor for the development of neural tube defects. However, recent meta-analyses show that folate supplementation in pregnancy might also lower the risk of other congenital malformations, such as orofacial clefts (Millacura et al. 2017) and congenital heart disease (Cheng et al. 2022). This is not surprising since uninterrupted folate metabolism plays a vital role in embryonic development, ensuring an ample supply of one-carbon activated folate cofactors for essential processes like de-novo purine and thymidine synthesis, the synthesis of the essential amino acid methionine, and the removal of the teratogenic amino acid homocysteine (Figure 1). Furthermore, the methionine cycle serves as the primary source of methyl donors, particularly S-adenosyl methionine, which is pivotal in numerous methylation reactions, including DNA methylation, a key regulatory mechanism for gene expression during embryogenesis.

There have been numerous studies researching the association of common polymorphisms in genes coding for 5,10-methylenetetrahydrofolatereductase (*MTHFR*) and reduced folate carrier (*RFC* or *SLC19A1*) with OFC and/or CHD. Products of both genes play a key role in the folate cycle, but studies of other genes of folate and methionine cycle are less numerous or even absent. What is more, studies on *MTHFR* and *SLC19A1* have produced ambiguous results and show high levels of heterogeneity. Two recent meta-analyses came to different conclusions considering the association of common *MTHFR* polymorphisms with OFC; (Zhou et al. 2020) found no association, but identified the protective role of folate supplementation in OFC prevention, whereas (Li et al. 2020) found that *MTHFR* polymorphism C677T is associated with OFC development. Considering CHD, a meta-analysis by (Mamasoula et al. 2013) has found that *MTHFR* polymorphism C677T shows an association with CHD, but only when small studies exhibiting a high level of heterogeneity are not excluded from the analysis. This means that the heterogeneity among the studies might be the cause for the ambiguous results. Interestingly, a meta-analysis by (Yi et al. 2021) found an association between the A80G polymorphism in the *RFC* gene and CHD development, but only when the polymorphism was present in mothers of affected children, but not in affected children. This indicates, that maternal genome can be equally or in some cases even more important in the etiopathology of congenital malformations than the fetal genome, which is more often investigated in research and diagnostic settings. This is confirmed in the recent meta-analysis by (Li et al. 2022) which investigated the association of *MTHFR* C677T with four neonatal defects (neural tube defects, OFC, CHD, and Down syndrome) in 81 444 subjects. The *MTHFR* polymorphism in mothers of affected children was associated with all four neonatal defects, whereas the same polymorphism in newborns was associated only with neural tube defects. This shows that the influence of the maternal genome on the development of various congenital defects is significant and should be considered when investigating the genetic markers of congenital malformations. The cause of ambiguous results of the previous studies might be due to studies comparing control and case children from different mothers (with different genetic makeup) to identify the genetic differences among children, with only some studies taking into account also the maternal genome. Thus, the more suitable study design would be the comparison of siblings with and without a birth defect from the same mother, thus minimizing the effect of the maternal genome. In this way, it might be possible to identify genetic markers of congenital malformations that pertain exclusively to the child.

In line with this, and to identify fetal genetic markers of OFC and CHD in folate and methionine cycles, we undertook a study of affected and unaffected sibling pairs, where the unaffected sibling serves as a control for the affected sibling. The advantage of this approach is that we compare the genetic differences between siblings with and without a birth defect that developed in the same in utero environment considering the maternal genetic makeup.

2 | Materials and Methods

2.1 | Study Cohort

The study cohort consisted of 122 pairs of children with OFC (OFC cases) and their siblings without OFC (OFC controls), and 115 pairs of children with CHD (CHD cases) and their siblings without CHD (CHD controls). The DNA from all children included in the study was collected using buccal swabbing. In addition, a questionnaire about the potential demographic and environmental risk factors during both affected (Cases with OFC/CHD) and unaffected (OFC/CHD control) pregnancies was completed by all mothers of the children in the study cohort. Cases with OFC (and their siblings without OFC) were recruited during routine follow-up examinations before or after the corrective surgery of their OFC at the Department of Maxillofacial and Oral Surgery at the University Clinical Center Ljubljana (UKCLJ). Cases with CHD (and their siblings without CHD)

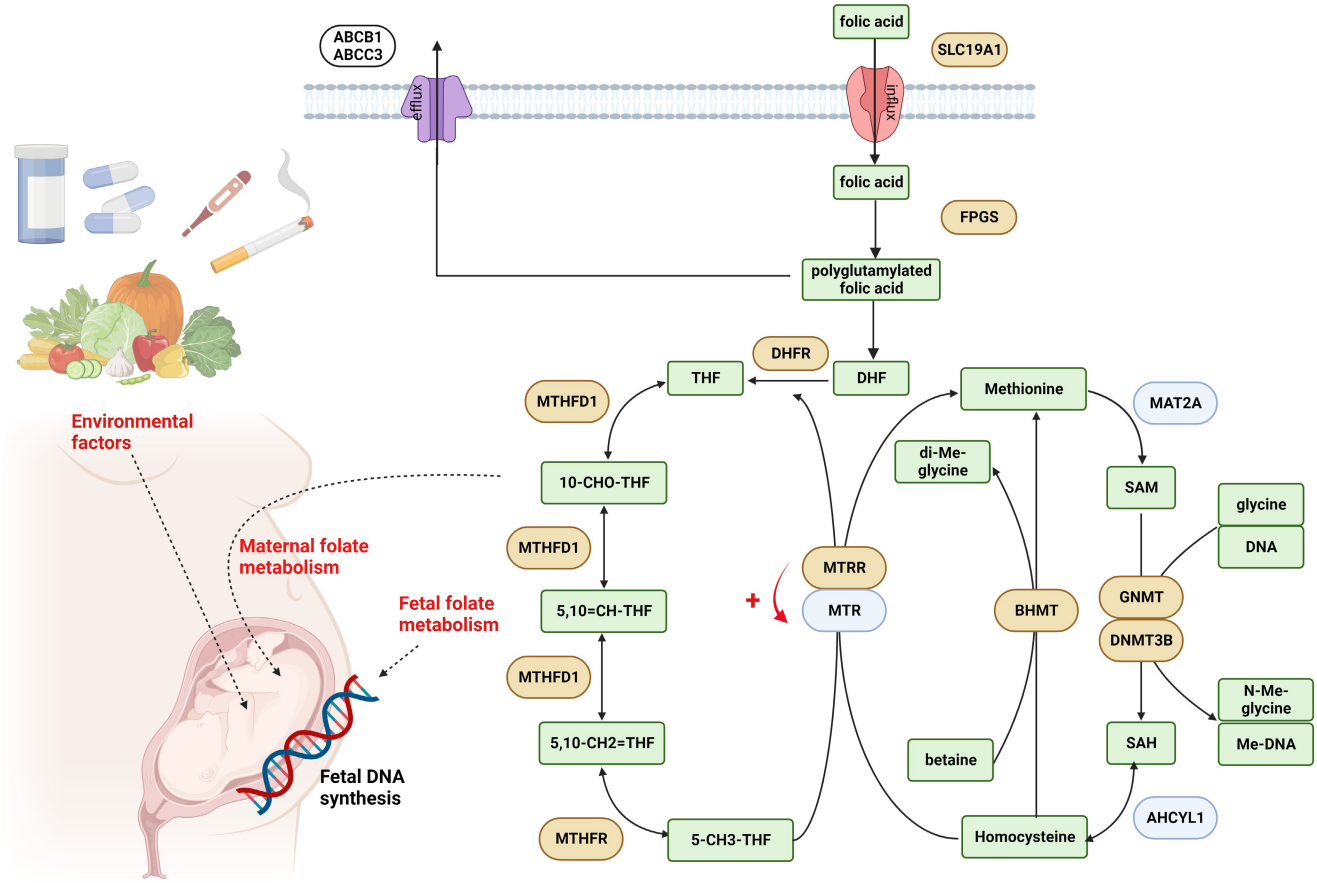

FIGURE 1 | Influence of genetic makeup and environmental factors affecting maternal and fetal folate metabolism on fetal development. Ellipses, enzymes and transporters; Rectangles, metabolites; Brown ellipses, enzymes and transporters investigated in this study. Abbreviation: 10-CHO-THF, 10-formyl tetrahydrofolate; 5,10=CH-THF, methenyl tetrahydrofolate; 5,10-CH2=THF, methylene tetrahydrofolate; 5-CH3-THF, 5-methyl tetrahydrofolate; ABCB1, P-glycoprotein; ABCC3, multidrug-resistant protein 3; ACHYL1, S-adenosylhomocysteine hydrolase-like 1; BHMT, betaine-homocysteine S-methyltransferase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; DNMT3B, DNA (cytosine-5-)-methyltransferase 3 beta; FPGS, foly-polyglutamyl synthase; GNMT, glycine N-methyltransferase; MAT2A, methionine adenosyl-transferase II alpha; Me, methyl; MTHFD1, trifunctional methylenetetrahydrofolate dehydrogenase/ cyclohydrolase/ synthase; MTR, 5-methyl tetrahydrofolate-homocysteine methyltransferase; MTRR, 5-methyl tetrahydro-folate-homocysteine methyltransferase reductase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SAM, S-adenosylmethionine; SLC19A1, solute-carrier family 19; THF, tetrahydrofolate.

were recruited at the Department of Cardiology, University Children's Hospital, University Medical Centre Ljubljana (Slovenia) during routine check-ups. In some cases, buccal swabs from the sibling without OFC or CHD were obtained by post. Informed consent was obtained from all of the participants and/or their legal guardians. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (NMEC) (No. 57/02/13) and was performed in accordance with the relevant guidelines and regulations.

2.2 | Evaluation of Environmental Risk Factors by Questionnaire

All mothers of each OFC or CHD sibling pair completed two identical questionnaires, one pertaining to the affected and one to the unaffected pregnancy. The questionnaire investigated known demographic and environmental OFC and CHD risk factors; thus, the following data were collected: child's birth year, maternal age at conception, maternal height and weight at conception, the sex of the child, maternal gestational diabetes, maternal medicine intake, maternal fever during the first trimester, and maternal folate supplementation before the conception and during pregnancy.

2.3 | DNA Extraction and Genotyping

The DNA was extracted from buccal swabs using QIAamp DNA mini kits (Qiagen) or MasterPure complete DNA and RNA purification kits (Epicentre, Illumina), following the manufacturer's instructions.

To assess interactions between the genes of interest, we conducted text and database mining utilizing STRING 10.0 (Szklarczyk et al. 2015). For each of the selected genes, we selected at least one polymorphism for genotype analysis. These polymorphisms exhibited a minor allele frequency of $\geq 25\%$ and had the highest number of PubMed connections to OFC and/or CHD and/or etiologically related conditions (e.g., NTD).

TABLE 1 | Comparison of the child's birth year, maternal age at conception, and maternal body mass index (BMI) at conception between the pregnancy with OFC and without OFC.

Risk factor	N^{a}	Pregnancy without OFC	Pregnancy with OFC[b]	p^{*}	p_{adj}^{**}
Birth year[c]	102	2006 (1987-2013)	2007 (1991-2014)	0.013	0.178
Maternal age at conception (years)[d]	102	27.8 ± 5.1	29.3 ± 4.5	0.009	0.135
Maternal body mass index at conception (kg/m^2)[c]	122	22.4 (16.2—36.2)	22.6 (16.6—37.0)	0.125	0.737

[a]Number of sibling pairs included in the analysis.
[b]Orofacial cleft.
[c]Non-Gaussian distribution (median (minimum-maximum)).
[d]Gaussian distribution (mean ± standard deviation).
*Paired samples *t*-test for Gaussian data, related-samples Wilcoxon signed rank test for non-Gaussian data.
**Adjusted *p* values (Holm-Sidak correction for multiple testing).

We analyzed ten common polymorphisms located in nine genes associated with the folate and methionine cycles. The analysis was performed using TaqMan (Applied Biosystems, Foster City, California, USA) or LightSNiP (TIB MOLBIOL, Berlin, Germany) probes, in accordance with the manufacturer's instructions. Specifically, the following polymorphisms were genotyped using TaqMan probes: rs1544105 (*FPGS*) (assay number C_8342611_10), rs1677693 (*DHFR*) (assay number C_3103231_10), rs1801133 and rs1801131 (*MTHFR*) (assay numbers C_1202883_20 and C_850486_20), rs1801394 (*MTRR*) (assay number C_3068176_10), rs2236225 (*MTHFD1*) (assay number C_1376137_10), rs3733890 (*BHMT*) (assay number C_11646606_20), rs10948059 (*GNMT*) (assay number C_11425842_10), and rs2424913 (*DNMT3B*) (assay number C_25620192_20). The genotyping of rs1051266 (*SLC19A1*) was conducted using LightSNiP assay.

2.4 | Statistical analysis

The normality of the distribution was checked using the Shapiro-Wilk test. To compare continuous variables between affected and unaffected siblings, a paired samples *t*-test was used for Gaussian data, and related-samples Wilcoxon signed rank test for non-Gaussian data. For categorical variables, related-samples McNemar test for the bi-categorical data, and related-samples marginal homogeneity test for multi-categorical data was used. Holm-Sidak correction for multiple testing was applied for adjustment of *p* values (p_{adj}).

For all genotypes, the additive, dominant, and recessive genetic models were calculated (Tables S1 and S2), but only the one with the highest statistical significance is presented in Tables 3 and 6.

All of the statistical analyses were performed using IBM SPSS Statistics 25.

3 | Results

To elucidate the influence of fetal folate metabolism on the development of congenital malformations (OFC and CHD), we compared genotypes of selected polymorphisms in the genes of folate–methionine cycles in pairs of OFC or CHD-affected and unaffected siblings. This study design minimized the influence of maternal folate metabolism, as affected and unaffected siblings in a sibling pair share the same mother, that is, very similar in utero conditions. In the same cohort, we also analyzed some demographic and environmental factors that might differ between sibling pregnancies and are known risk factors for congenital malformations. Of 122 OFC sibling pairs, 45 had DNA samples available for genotype analysis (44 pairs on all 10 investigated loci), whereas 31 of 115 CHD sibling pairs were available for DNA sampling and analysis.

3.1 | Analysis of OFC sibling pairs

To identify demographic and environmental risk factors of OFC, we investigated 122 sibling pairs, where one of the siblings had isolated non-syndromic OFC. Data about the investigated

demographic and environmental risk factors were available for all sibling pairs, except for birth year and maternal age at conception, which were available for 102 sibling pairs. Comparisons of demographic and environmental risk factors between OFC-affected and -unaffected siblings, where the unaffected sibling serves as a control, are shown in Tables 1 and 2. Higher birth years and more advanced maternal age at conception were associated with the increased risk of OFC development. Still, these associations did not stay statistically significant after the correction for multiple testing (Table 1). The only environmental factor that was significantly associated with OFC after the Holm-Sidak correction was the presence of maternal fever in the 1st trimester of pregnancy (p_{adj}=0.035). Fever (a body temperature of 38.5°C or more, lasting for 2 days or more) was present in 16.4% of pregnancies with OFC, but only 3.3% of unaffected pregnancies (Table 2).

Next, genotypes at 10 loci in 9 genes of folate–methionine cycles were compared between siblings with OFC and without OFC in 45 sibling pairs to identify fetal genetic risk factors for OFC. For each genotype, the comparison was made in three genetic models (additive, dominant, and recessive) (Table S1). For each genotype, the model with the lowest *p*-value was chosen for Sidak-Holm adjustment for multiple testing. Only four genotypes in three genes (*MTHFR* c.677 C>T, *MTHFR* c.1298 A>C, *MTHFD*1 c.1958 G>A, and *GNMT* g.4962 C>T) were associated with OFC (Table 3). However, after the adjustment for multiple testing, none of the genetic factors was significantly associated with OFC (Table 3).

We also compared the total number of variant alleles at 10 loci between siblings with OFC and without OFC in 44 OFC pairs. The total number of variant alleles in 9 genes of folate/methionine cycles was significantly higher in children with OFC (median: 12), compared to their unaffected siblings (median: 9, $p < 0.001$) (Figure 2A).

3.2 | Analysis of CHD sibling pairs

To identify demographic and environmental risk factors of OFC, we investigated 115 sibling pairs, where one of the siblings had isolated non-syndromic CHD. Data for the following demographic and environmental risk factors were not available for all of 115 sibling pairs: birth year (available for 108 pairs), Maternal BMI at conception (available for 113 pairs), child's gender (available for 114 pairs), and folate supplementation during pregnancy (available for 112 pairs). Comparisons of demographic and environmental risk factors between siblings with and without CHD, where unaffected sibling serves as a control, are shown in Tables 4 and 5. After the correction for multiple testing, four demographic and environmental factors were associated with CHD. Children with CHD were born in a later birth year than their unaffected siblings ($p < 0.001$) (Table 4). Mothers were on average 3 years older at the time of the birth of the child with CHD compared to the birth of the unaffected child ($p = 0.0033$) (Table 4). Furthermore, mothers took the medicines more often during the

TABLE 2 | Comparison of the environmental risk factors between the pregnancy with and without OFC.

Risk factor	*N*[a]	Pregnancy without OFC	Pregnancy with OFC[b]	*p**	p_{adj}**
Child's gender	122			0.156	0.783
Male		58 (47.5%)	70 (57.4%)		
Female		64 (52.5%)	52 (42.6%)		
Gestational diabetes	122			0.388	0.947
No		115 (94.3%)	111 (91.0%)		
Yes		7 (5.7%)	11 (9.0%)		
Medicine consumption in the 1st trimester	122			0.029	0.298
No		87 (71.3%)	72 (59.0%)		
Yes		35 (28.7%)	50 (41.0%)		
Fever in 1st trimester	122			0.002	0.035
No		118 (96.7%)	102 (83.6%)		
Yes		4 (3.3%)	20 (16.4%)		
Folate supplementation during pregnancy	122			0.710	0.974
Earlier than 3 weeks post-conception		57 (46.7%)	55 (45.1%)		
Later than 3 weeks post-conception		15 (12.3%)	22 (18.0%)		
None		50 (41.0%)	45 (36.9%)		

[a]Number of sibling pairs included in the analysis.
[b]Orofacial cleft.
*Related-samples McNemar test for the bi-categorical data, related samples marginal homogeneity test for multi-categorical data.
**Adjusted *p* values (Holm-Sidak correction for multiple testing).

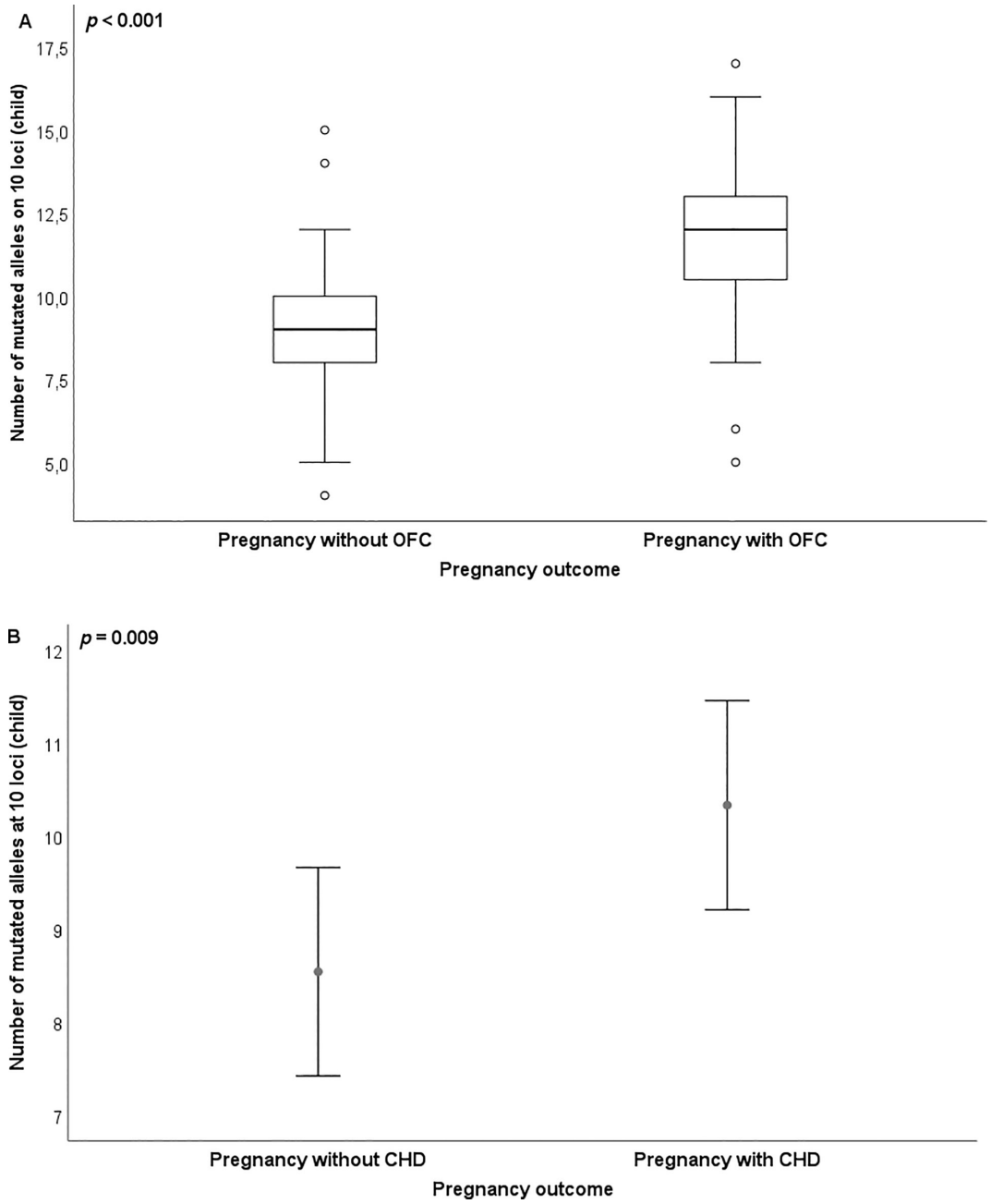

FIGURE 2 | Total number of variant alleles at 10 loci in a child: A comparison between siblings with and without a birth defect in 44 OFC (A) and 24 CHD (B) sibling pairs. (A) A comparison between siblings without OFC (median (minimum-maximum): 9 (4–15)) and siblings with OFC (median (minimum-maximum): 12 (5–17)) ($p < 0.001$, Wilcoxon matched-pair signed-rank test). Boxplots represent the median and interquartile range. (B) A comparison between siblings without CHD (mean ± SD: 8.5 ± 2.7) and siblings with CHD (mean ± SD: 10.3 ± 2.7) ($p = 0.009$, Paired samples *t*-test). Error bars represent the mean and 95% confidence interval. Abbreviation: CHD, congenital heart disease; OFC, orofacial cleft.

pregnancy that resulted in the birth of a child with CHD (56.5% of mothers) than during the unaffected pregnancy (35.7% of mothers, $p = 0.003$) (Table 5). There was also a significant difference in maternal folate supplementation during affected and unaffected pregnancies ($p = 0.015$), but surprisingly mothers started taking the folate supplement earlier during the CHD-affected pregnancy than during the unaffected pregnancy (Table 5).

Genotypes at 10 loci in 9 genes of folate–methionine cycles were compared between siblings with CHD and siblings without CHD in 31 sibling pairs to identify fetal genetic risk factors for CHD. For each genotype, the comparison was made in three genetic models (additive, dominant, and recessive) (Table S2). For each genotype, the model with the lowest p-value was chosen for Sidak-Holm adjustment for multiple testing. Four genotypes in four genes (*BHMT* c.716 G>A, *DNMT3B* g.29069C>T, *FPGS* g.2572C>T, and *DHFR* g.19483C>A) were associated with CHD (Table 6). However, after the adjustment for multiple testing, only *FPGS* g.2572C>T homozygous variant genotype (TT) was significantly associated with CHD. Prevalence of the TT genotype was significantly higher in CHD children (51.6%) compared to their unaffected siblings (12.9%, $p = 0.021$) (Table 6).

TABLE 3 | Comparison of the *MTHFR, MTHFD1,* and *GNMT* genotypes between the sibling with OFC and the sibling without OFC.

Child's genotype	*N*[a]	Child without OFC[b]	Child with OFC[b]	*p**	p_{adj}**
MTHFR c.677 C>T (additive genetic model)	45			0.017	0.213
CC		21 (46.7%)	13 (28.9%)		
CT		13 (28.9%)	25 (55.6%)		
TT		11 (24.4%)	7 (15.6%)		
MTHFR c.1298 A>C (additive genetic model)	45			0.004	0.066
AA		31 (68.9%)	23 (51.1%)		
AC		4 (8.9%)	17 (37.8%)		
CC		10 (22.2%)	5 (11.1%)		
MTHFD1 c.1958 G>A (additive genetic model)	45			0.021	0.241
GG		15 (33.3%)	10 (22.2%)		
AG		15 (33.3%)	26 (57.8%)		
AA		15 (33.3%)	9 (20.0%)		
GNMT g.4962 C>T (additive genetic model)	45			0.035	0.324
CC		6 (13.3%)	13 (28.9%)		
CT		31 (68.9%)	24 (53.3%)		
TT		8 (17.8%)	8 (17.8%)		

[a]Number of sibling pairs included in the analysis.
[b]Orofacial cleft.
*Related-samples McNemar test for the bi-categorical data, related samples marginal homogeneity test for multi-categorical data.
**Adjusted *p* values (Holm-Sidak correction for multiple testing).

Lastly, we compared the total number of mutated alleles at 10 loci between siblings with and without CHD in 24 CHD pairs. The total number of variant alleles in 9 genes of folate/methionine cycles was significantly higher in children with CHD (mean: 10.3), compared to their siblings without CHD (mean: 8.5, $p = 0.009$) (Figure 2B).

4 | Discussion

Folates have been long known or suspected to modulate the development of several congenital malformations. However, while the association with some congenital malformations is clear (i.e., neural tube defects), the connection to others (OFC, CHD) is still uncertain, since nutritional and genetic studies have given ambiguous results. To identify risk factors of OFC and CHD, we used a novel approach by comparing demographic, environmental, and genetic factors between children affected with OFC or CHD and their unaffected siblings. Since sibling pairs share more similar in-utero environment than unrelated cases and controls, the fetal genetic factors for congenital malformations might be more clearly defined and identified. Sibling pairs share similar, but not identical *in utero* environment, since maternal genetic makeup (and consequently metabolism) is identical, but demographic and environmental factors might differ between sibling pregnancies. Therefore, in addition to the fetal genetic factors influencing folate metabolism, we also studied some known demographic and environmental risk factors.

We identified only one environmental risk factor for OFC, maternal fever in the first trimester of pregnancy. Our results are in line with (Shahrukh Hashmi et al. 2010) who undertook a large study on 5821 controls and 2402 cases of orofacial clefts. They found that maternal fever (especially if not controlled with antipyretics) was associated with the increased risk of orofacial clefts, specifically cleft lip with or without cleft palate. The meta-analysis by (Luteijn, Brown, and Dolk 2014), including 29.542 infants, concluded that the first-trimester maternal fever was associated with an increased risk of any congenital anomaly [adjusted odds ratio (AOR) 2.00, 95% CI: 1.62–2.48], including cleft lip (AOR: 3.12, 95% CI: 2.20–4.42). A recent meta-analysis by (Shi et al. 2023) of 13 case-control studies identified maternal fever as a modest but statistically significant risk factor for cleft lip with or without cleft palate (AOR: 1.91, 95% CI: 1.3–2.8).

Considering fetal genetic risk factors determining folate/methionine metabolism, none of the selected polymorphisms was associated with the increased risk of OFC in this study. However, the total number of variant alleles in 9 genes related to the folate/methionine cycles was significantly higher in children with

TABLE 4 | Comparison of the child's birth year, maternal age at conception, and maternal BMI at conception between the pregnancy with CHD and without CHD.

Risk factor	N[a]	Pregnancy without CHD	Pregnancy with CHD[b]	p*	p_{adj}**
Birth year[c]	108	2010 (1987-2015)	2012 (1996-2015)	3.5×10^{-6}	6.3×10^{-5}
Maternal age at conception (years)[d]	115	27.1 ± 5.4	30.1 ± 4.8	1.9×10^{-4}	3.3×10^{-3}
Maternal body mass index at conception (kg/m^2)[c]	113	22.6 (17.0 – 184)	23.0 (17.0 – 44.0)	0.010	0.114

[a]Number of sibling pairs included in the analysis.
[b]Congenital heart disease.
[c]Non-Gaussian distribution (median (minimum-maximum)).
[d]Gaussian distribution (mean ± standard deviation).
*Paired samples *t*-test for Gaussian data. related-samples Wilcoxon signed rank test for non-Gaussian data.
**Adjusted *p* values (Holm-Sidak correction for multiple testing).

OFC compared to their unaffected siblings. This suggests a multifactorial inheritance pattern, where the combined influence of multiple genes (not limited to just 9 investigated genes) contributes to the development of the OFC, even though the effects of individual genes may be relatively modest. However, some studies of unrelated cases and controls have associated the variants in genes of folate/methionine cycles with increased risk of OFC, although with a low level of confidence. Selected genes of folate/methionine cycles indicated in OFC development by other researchers in non-related case-control studies are reviewed in Table S3.

Four demographic and environmental risk factors for CHD were identified in the present study: birth year, maternal age at conception, medicine consumption during 1st trimester, and maternal folate supplementation during pregnancy. On average, the cases with CHD were born at later birth years and to older mothers than their siblings without CHD. Our results are in line with the results of (Luo et al. 2013) which identified the advanced maternal age as a risk factor for CHD. Another recent study found that different subclasses of CHD are differentially influenced by maternal age, as atrial septal defects are more common in babies of younger mothers, while babies of older mothers present mostly with ventricular septal defects and patent ductus arteriosus (Hashim Jr. et al. 2020). On the other hand, some studies have found no association between maternal age and CHD in offspring (Best and Rankin 2016), which might be due to the etiological heterogeneity of CHD subtypes. Furthermore, in the present study the medicine consumption was more common during the pregnancy with CHD (57%) than during the pregnancy that resulted in the birth of a sibling without CHD (36%). Of note, the consumption of all medicines was considered for the data analysis, except for the antiepileptics, which were taken by none of the mothers, neither during pregnancy with CHD nor during pregnancy without CHD. Several classes of medications have been identified as risk factors for CHD and other congenital malformations, although the results of different studies have been inconsistent and non-reproducible. Following medication classes have been associated with the various types of CHD: antihypertensives (angiotensin-converting enzyme inhibitor, antiadrenergic, β-blockers, Ca channel blockers, diuretics), antibacterial drugs (sulfonamides, macrolides), insulin, fertility drugs (clomiphene, chorionic gonadotropin), nonsteroidal anti-inflammatory drugs (naproxen), antiepileptics, antidepressants (selective serotonin reuptake inhibitors, tricyclic antidepressants), and other classes of psychotropic medication (lithium, benzodiazepines) (Boyd et al. 2022). Our results on the influence of folate supplementation during pregnancy on CHD development that the greater proportion of mothers had early folate supplementation during pregnancy with CHD (41%), compared to the pregnancy without CHD (23%), were somewhat surprising since the majority of other studies identified the lack of folic acid supplementation as a risk factor for CHD (Csaky-Szunyogh et al. 2014; Vereczkey et al. 2013; Yan et al. 2022; Zhong et al. 2022). However, in some animal (Mikael et al. 2013) and human (Leirgul et al. 2015) studies the increased maternal folate intake in the periconceptional period was associated with the increased risk of septal defects in offspring. This was also confirmed in a recent meta-analysis that found that overall maternal folate supplementation was associated with a decreased risk of CHD, but with a high level of heterogeneity among the studies. What is more, the meta-analysis concluded that folate supplementation within 1 month before and

TABLE 5 | Comparison of the environmental risk factors between the pregnancy with CHD and without CHD.

Risk factor	N[a]	Pregnancy without CHD	Pregnancy with CHD[b]	p*	p_{adj}**
Child's gender	114			0.221	0.770
Male		56 (49.1%)	66 (57.9%)		
Female		58 (50.9%)	48 (42.1%)		
Gestational diabetes	115			0.065	0.454
No		107 (93.0%)	100 (87.0%)		
Yes		8 (7.0%)	15 (13.0%)		
Medicine consumption in the 1st trimester	115			0.0002	0.0033
No		74 (64.3%)	50 (43.5%)		
Yes		41 (35.7%)	65 (56.5%)		
Fever in 1st trimester	115			0.375	0.770
No		113 (98.3%)	110 (95.7%)		
Yes		2 (1.7%)	5 (4.3%)		
Folate supplementation during pregnancy	112			0.001	0.015
Earlier than 3 weeks post-conception		26 (23.2%)	46 (41.1%)		
Later than 3 weeks post-conception		48 (42.9%)	33 (29.5%)		
None		38 (33.9%)	33 (29.5%)		

[a]Number of sibling pairs included in the analysis.
[b]Congenital heart disease.
*Related-samples McNemar test for the bi-categorical data, related samples marginal homogeneity test for multi-categorical data.
**Adjusted *p* values (Holm-Sidak correction for multiple testing).

TABLE 6 | Comparison of the *BHMT, DNMT3B, FPGS,* and *DHFR* genotypes between children with CHD and their siblings without CHD.

Child's genotype	N[a]	Child without CHD[b]	Child with CHD[b]	p*	p_{adj}**
BHMT c.716 G>A (recessive genetic model)	29			0.007	0.0873
GG or AG		24 (82.8%)	13 (44.8%)		
AA		5 (17.2%)	16 (55.2%)		
DNMT3B g.29069C>T (recessive genetic model)	30			0.013	0.134
CC or CT		24 (80.0%)	14 (46.7%)		
TT		6 (20.0%)	16 (53.3%)		
FPGS g.2572C>T (recessive genetic model)	31			0.0015	0.0208
CC or CT		27 (87.1%)	15 (48.4%)		
TT		4 (12.9%)	16 (51.6%)		
DHFR g.19483C>A (recessive genetic model)	29			0.039	0.328
CC or CA		28 (96.6%)	21 (72.4%)		
AA		1 (3.4%)	8 (27.6%)		

[a]Number of sibling pairs included in the analysis.
[b]Congenital heart disease.
*Related-samples McNemar test for the bi-categorical data, related samples marginal homogeneity test for multi-categorical data.
**Adjusted *p* values (Holm-Sidak correction for multiple testing).

after conception correlated positively with CHD and high-dose folic acid intake was positively associated with atrial septal defect (Cheng et al. 2022).

Considering fetal genetic risk factors determining folate/methionine metabolism, the polymorphism in the *FPGS* gene was associated with the increased risk of CHD in this study. More precisely, the homozygous variant genotype TT was associated with an increased risk of CHD ($p_{adj} = 0.021$). To date, no studies investigated the association between FPGS and CHD, except for our previous family triads (mother-father-child) study that found the association between the *FPGS* polymorphism and left ventricular outflow tract obstruction (LVOTO) development using likelihood ratio analysis (Karas Kuzelicki et al. 2022). The *FPGS* gene encodes the enzyme folylpolyglutamate synthase which is located mainly in cytoplasm and mitochondria. This enzyme adds glutamate moieties to folate-monoglutamates that are commonly present in the blood. Polyglutamation of folates is of key importance for the maintenance of their intracellular concentrations, as only monoglutamate forms of folates and folate derivatives can enter and/or leave the cell through influx and/or efflux transporters, respectively. Thus reduced activity of FPGS would result in decreased intracellular levels of folates (Kim 2020). Reduced intracellular levels of folates are problematic during fetal development since they can lead to various congenital malformations. So far the variants in the maternal *FPGS* gene have been associated with orofacial clefts in offspring (Inostroza et al. 2021). *FPGS* was more extensively studied in the scope of pharmacogenomics of some anticancer drugs (Huang et al. 2016), such as methotrexate and 5-fluorouracil.

As in the case of OFC, we found an increased number of variant loci in 9 genes of folate/methionine cycles in children with CHD compared to their siblings without CHD, indicating additive small-effect influences from several genes. Selected genes of folate/methionine cycles indicated in CHD development by other researchers in non-related case-control studies are reviewed in Table S4. The main limitation of the study is the low number of cases of OFC and CHD with unaffected siblings and the consequent need to collapse subtypes for each defect group. In the case of a bigger sample size and the analyses of specific subtypes, the genetic determinants for specific OFC and CHD subtypes might be identified.

5 | Conclusions

In this uniquely designed study of environmental and genetic factors influencing the risk of OFC and CHD, we investigated pairs of affected and unaffected siblings, thus minimizing the effect of maternal metabolism.

A comparison of children with OFC and their unaffected siblings revealed that only the presence of maternal fever in the first trimester of pregnancy increased the risk of OFC formation.

On the other hand, when comparing CHD cases with their unaffected siblings, we found that later birth year, higher maternal age at conception, taking any medicine in the first trimester of pregnancy, and the homozygosity for the g.2572C>T polymorphism in the *FPGS* gene increased the risk of CHD formation.

Interestingly, both OFC and CHD formation were associated with a higher number of mutated loci in genes of folate–methionine cycles, indicating polygenic and possibly multifactorial inheritance.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.