"Association of MTHFR and MS/MTR gene polymorphisms with congenital heart defects in North Indian population (Jammu and Kashmir): a case–control study encompassing meta-analysis and trial sequential analysis"

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Abstract
Background: The risk of Congenital Heart Defects (CHD) is greatly influenced by variants within the genes involved in folate-homocysteine metabolism. Polymorphism in MTHFR (C677T and G1793A) and MS/MTR (A2756G) genes increases the risk of developing CHD risk, but results are controversial. Therefore, we conducted a case–control association pilot study followed by an up-dated meta-analysis with trial sequential analysis (TSA) to obtain more precise estimate of the associations of these two gene variants with the CHD risk.

Methods: For case–control study, we enrolled 50 CHD patients and 100 unrelated healthy controls. Genotyping was done by PCR–RFLP method and meta-analysis was performed by MetaGenyo online Statistical Analysis System software. For meta-analysis total number of individuals was as follows: for MTHFR C677T 3450 CHD patients and 4447 controls whereas for MS A2756G 697 CHD patients and 777 controls.

Results: Results of the original pilot study suggested lack of association for MTHFR C677T and MS A2756G polymorphism with risk of CHD whereas MTHFR G1793A was significantly associated with the disease. On performing meta-analysis, a significant association was observed with MTHFR C677T polymorphism but not with MS A2756G. Trial sequential Analysis also confirmed the sufficient sample size requirement for findings of meta-analysis.

Conclusions: The results of the meta-analysis suggested a significant role of MTHFR in increased risk of CHD.

Keywords: Polymorphism, MTHFR, Met-analysis, TSA

Introduction
Congenital heart diseases or defects (CHD) which share a significant proportion in CVD burden arises due to incomplete development of heart during the first 6-weeks of gestation [1]. The origin of CHD is diverse which can be associated with a syndrome or be isolated (non-syndromic). It is hypothesized that susceptibility of cardiac
defects increases with dual interaction of key gene(s)/SNP-environmental factors which perturb normal cardiac developmental process during embryonic life. The risk of CHD is greatly influenced by variants within the genes involved in folate-homocysteine metabolism [2–4]. Many studies have revealed that the risk of CHD in newborns of females carrying mutations in genes involved in folate metabolism can be reduced by maternal periconceptional use of multivitamins or folic acid [5], however, the mechanism underlying this effect is still under investigation. Folate and vitamin B12 are known to influence homocysteine concentration. Folates taken in diet are usually polyglutamates which are converted to simpler forms, particularly monoglutamates, dihydrofolate, tetrahydrofolate and finally to methylated form of folate i.e. 5, 10-methylenetetrahydrofolate (5, 10-MTHF) and 5-methyltetrahydrofolate (5-MTHF) by a specialised enzyme of the pathway. Homocysteine and folate metabolism is dependent on a couple of genes performing their specific role but two genes namely MTHFR and MS are considered critical genes for development of diseased cardiovascular phenotypes. A common mutation, C677T (rs1801133), in exon 4 of the MTHFR gene results in decreased enzyme activity and contributes to increased plasma homocysteine, particularly in individuals with low folate status. Rady and co-workers reported a novel polymorphic site of the MTHFR gene at nucleotide position 1793 G to A transition in exon 11 (rs22396976) which results an arginine-to-glutamine change at codon 594 and modifies enzyme activity [6]. The A2756G mutation (rs1805087) in MS gene alters re-methylation process and is also associated with increased homocysteine levels and risk of CHD. Most of the research in relation to folate-homocysteine metabolising pathway with the risk of CHD is based on parent-of-origin effect. There are very few studies focussing on embryonic variation in candidate genes of folate-homocysteine metabolising pathway in association with the development of structural congenital heart malformations during early pregnancy. Consistent with this view, we attempted to perform a case–control pilot study involving evaluation of two important genes: MTHFR (C677T and G1793A) and MS (A2756G) gene variations with risk of CHD in Jammu region of UT of J&K, India. Further, we also performed an updated meta-analysis with trial sequential analysis to investigate the association between MTHFR (C677T and G1793A) and MS (A2756G) polymorphisms and risk of CHD with increased statistical power.

Methodology

Study population and area

The present study was ethically approved by Institutional Ethical Committee, University of Jammu. The present study was carried out on 150 children, out of whom 50 children (0–12 years) were confirmed cases of CHD and 100 children (below 18 years) were unrelated healthy controls belonging to Jammu region of Union Territory of Jammu and Kashmir. The CHD cases were enrolled from In-patient Department of Paediatrics whereas controls were recruited from Out-patient Department of Paediatrics, Shri Maharaja Gulab Singh (SMGS) hospital, Jammu. Data and blood collection was done after having an informed written consent from attendant or guardian of the children. The diagnosis and classification of CHD was based on the clinical and the echocardiography findings. The inclusion/exclusion criteria were followed wherein patients with any form of CHD were included whereas patients with syndromes and neural tube defects were excluded. Controls admitted to hospital for minor ailments with no history of CHD or other major abnormality and also children visiting for blood typing were recruited for the study under reference. Power of the study for sample size calculation was done by using online tool based on mean and standard deviation of two groups of study subjects, two tail test and with alpha value of 5% (https://www.sphanalytics.com/statistical-power-calculator-using-average-values/). The power of the study obtained was more than 80%.

Blood collection and DNA isolation

500 μl-1 ml of blood was collected in EDTA coated vacu- tainers from each child by trained paramedical staff of the Hospital. Isolation of DNA from whole blood was carried out using commercially available kits (DNeasy Blood and Tissue Kit, QIAGEN). The quantitative and qualitative analysis of isolated DNA was performed by spectrophotometry and 1.5% agarose gel electrophoresis respectively.

Genotyping

Genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) technique. Briefly, PCR was carried out in a reaction volume of 25 μl each in thin walled tubes, consisting of 5.0 μl of PCR buffer (10X), 2.5 μl of MgCl2 (25 mM), 0.5 μl of dNTPs (10 mM), 0.5 μl (100 pmol/μl) of each of the forward and reverse primers, 0.3 μl (5unit/μl) of Taq DNA polymerase enzyme and 2 μl (40 ng) of genomic DNA. PCR amplification was carried out using the Veriti, Applied Biosystems by life technology, Singapore and amplification and RFLP conditions for all the three polymorphisms are given in Table 1. The gel images of PCR–RFLP for MTHFR (C677T and G1793A) and MS (A2756G) polymorphisms with band sizes have been depicted in Fig. 1, 2 and 3 respectively.
Statistical analyses

Genotypic frequency as well as allelic frequency was calculated by gene counting method. Hardy–Weinberg equilibrium (HWE) analysis and the differences in genotypic frequencies between two study groups were examined by using Pearson's goodness of fit Chi-square test. To assess the association, odds ratios (OR) with 95% CI were calculated under different genetic models by using Statistical Package for Social Sciences (SPSS-version 20) software and also by another method provided by the Institute of

Table 1  Details of Primer sequence, amplification conditions and restriction enzymes

| Gene polymorphism | Primer sequence | Amplicon (bp) | PCR conditions | Restriction enzymes | Genotypes | Reference |
|-------------------|-----------------|--------------|----------------|---------------------|------------|-----------|
| MTHFR C677T (rs1801133) | 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' (F) 5'-AGG ACG GTG CGG TGA GAG TG-3' (R) | 198 | Pre-Denaturation: 94 °C/ 2 min Denaturation: 94 °C/ 30 s Annealing: 62 °C/ 30 s Extension: 72 °C/ 7 min (40 cycles) | Hinfl | CC = 198 bp CT = 198, 175 & 23 bp TT = 175 & 23 bp | McBride et al., 2004 [36] |
| MTHFR G1793A (rs2274976) | 5'-CTC TGT GTG TGT GTG CAT GTG TGC G-3' (F) 5'-GGG ACA GGA GTG GCT CCA ACG CAG G-3' (R) | 310 | Pre-Denaturation: 94 °C/ 1 min Denaturation: 94 °C/ 1 min Annealing: 67 °C/ 1 min Extension: 72 °C/ 1 min Final Extension: 72 °C/ 7 min (40 cycles) | BsrBI | GG = 233 & 77 bp GA = 310, 233 & 77 bp AA = 310 bp | Rady et al., 2002 [6] |
| MS A2756G (rs185087) | 5'- TGT TCC AGA CAG TTA GAT GAA AAT C-3' (F) 5'- GAT CCA AAG CCT TTT ACA CTC CTC-3' (R) | 211 | Pre-Denaturation: 95 °C/ 4 min Denaturation: 95 °C/ 1 min Annealing: 61 °C/ 1.5 min Extension: 72 °C/ 1 min Final Extension: 72 °C/ 7 min (35 cycles) | HaeIII | AA = 211 bp AG = 211, 131 & 80 bp GG = 131 & 80 bp | Sahiner et al., 2014 [25] |

Fig. 1  RFLP Gel Image of MTHFR C677T Polymorphism. L: 100 bp Ladder; Lane 1,2,3,5,6,8: 198 bp (CC: Wild); Lane 4,7: 198 + 175 + 23 bp (CT: Hetero). *23 bp band will not be visible on agarose gel
Human Genetics accessed via the link: http://ihg.gsf.de/cgi-bin/hw/hwa1.pl. A $p$-value of $< 0.05$ was considered as statistically significant.

**Meta-analysis**

**Literature search**

Research papers (published up to February, 2021) examining the association between *MTHFR* C677T, *MTHFR* G1793A and *MS/MTR* A2756G polymorphisms and congenital heart defects were extracted from databases such as PubMed, Science direct, Proquest, Ovid and Google Scholar. Key words used for the database search were as follows: methylenetetrahydrofolate reductase; *MTHFR* gene polymorphisms; Methionine synthase; *MS/MTR* gene polymorphisms; Congenital heart defects; Congenital heart diseases; *MTHFR* C677T; *MTHFR* G1793A and *MS/MTR* A2756G. Reference records of studies included in our meta-analysis were manually searched for possible eligible articles.
**Inclusion and exclusion criteria**
The inclusion/exclusion criteria used for screening of eligible study are given in Table 2.

**Data extraction and quality assessment**
From each eligible study, the following data were extracted by the two investigators independently using a standardized form: first author, publication year, country of origin, ethnicity, number of cases and controls, genotype frequency, source of controls, genotyping method, and Hardy–Weinberg equilibrium (HWE). We investigated the quality of each study based on the nine-point Newcastle–Ottawa Scale (NOS). The characteristics and results of NOS for all the included studies are shown in Table 3. The NOS scores for all eligible studies in this Meta analysis exceeded 6 points, indicating that our analysis is updated and of good quality.

**Statistical analysis for meta-analysis**
The association between the selected polymorphisms and congenital heart defects was evaluated for each study by the crude odds ratios (ORs) with 95% confidence intervals (CIs). For each study, HWE was assessed by the chi-square goodness of fit test. For all studies, we estimated the association under three different genetic models [Allele contrast, dominant model and recessive model]. Statistical heterogeneity between studies was assessed by Cochran’s Q test and I-square ($I^2 > 50\%$) indicated the significance [31]. When $I^2 > 50\%$, a random-effect model should be taken otherwise fixed model is used. To calculate the OR and draw inference for each study, we used both random effects model and fixed effect model. Sensitivity analyses were conducted by omitting any single study, which predisposed the observed heterogeneity excessively and there should be no change in OR’s. Egger’s test and Begg’s funnel plot is used to solve the problem of Publication bias. All statistical analyses were performed in the MetaGenyo online Statistical Analysis System software [32].

**Trial sequential analysis (TSA)**
Meta-analysis may result in Type I error owing to an increased risk of random errors (play of chance) which can be due to dispersed data and repeated significance testing. Bias from low trial with low methodological qualities, publication bias and small trial bias may result in false $p$-value. Trial Sequential analysis is a methodology that can be used in meta-analysis to control random errors, and to assess whether the studies included in the meta-analysis have surpassed the requisite sample size. TSA was performed to calculate the required information size on the basis of overall 5% risk of Type-I error and a power of 80% for checking the reliability of meta analysis [33].

**Results**

**Case–control study**
Based on echocardiography reports, the different CHD phenotypes were categorised (Table 4). The observed prevalence of different CHD phenotypes in present study was highest for ventricular septal defect (VSD: 34%) and atrial septal defect (ASD: 26%) followed by tetralogy of fallot (TOF: 14%) and patent ductus arteriosus (PDA: 8%) and least for endocardial cushion defect (6%). The frequency of complex CHD forms (more than one CHD condition) were as follows: 4% for ASD with PDA, 2% for VSD with AV-canal defect, 4% for VSD with pulmonary arterial hypertension (VSD-PAH) and 2% for endocardial cushion defect along with dextrocardia.

The genotypic and allelic frequencies along with Chi square values for Hardy–Weinberg calculations for the all the three polymorphisms in study participants are depicted in Table 5. There observed frequencies of genotypes were in concordance with HWE in both the groups for all the polymorphisms except for MTHFR C677T in patient group. The genotypic frequency of CC, CT and TT (MTHFR C677T) in CHD patients was 88%, 8% and 4% whereas in controls it was 90%, 9% and 1% respectively. The frequency of variant allele T (0.08) was higher in CHD patients than controls (0.05) whereas wild allele C was reported to be in slightly higher frequency in controls (0.95) as compared to patients (0.92). The genotypic frequencies for MTHFR G1793A in CHD patients were 58%, 38% and 4% for GG, GA and AA respectively. The frequencies in control group were 90% for GG and 10% for

| Table 2  | Inclusion/Exclusion criteria for eligible studies |
|---------|-----------------------------------------------|
| **Studies included** | **Studies excluded** |
| ● Studies with Case–control designs | ● Case reports |
| ● Report of the association between the MTHFR C677T, MTHFR G1793A and MS A2756G polymorphism and the risk of CHD | ● Meta analysis and review articles |
| ● Studies that included Pediatric participants | ● Studies without control group |
| ● Studies that follow Hardy–Weinberg equilibrium (HWE) | ● Studies with abstract only |
| ● Studies with sufficient data | ● Studies that include maternal/ paternal cases only |
| ● Studies in English language | ● Studies without detailed genotype data |
| | ● Studies that are associated with other diseases like CVD’s, thrombosis, coronary artery defects etc |
### Table 3  Characteristics of the included studies in the meta-analysis

| Study                  | Age Group/ Mean age of cases | Mean age of controls | Diagnostic criteria                                                                 | Source of controls | Country/ Region | Ethnicity | Genotyping Method | Cases   | Controls | NOS | HWE  |
|------------------------|------------------------------|----------------------|--------------------------------------------------------------------------------------|-------------------|-----------------|-----------|-------------------|---------|----------|-----|------|
| Junker et al, 2001 [9] | 0–16                         | Age matched          | Echocardiography excluding DS or Chromosomal abnormality                              | HB                | Germany         | Caucasian | PCR-RFLP          | 51      | 129      | 21  | 228  | 0.0751 |
| Lee et al, 2005 [10]   | Children                     | -                    | Confirmed CHD patients for cardiac catheterization                                   | HB (cord blood from healthy foetuses) | Taiwan         | Asian     | DHPLC             | 110     | 114      | 14  | 213  | 0.5128 |
| Li et al, 2005 [11]    | Children                     | Age matched          | Registered patients of birth defects confirmed for CHD                              | HB                | China           | Asian     | PCR-RFLP          | 30      | 22       | 57  | 103  | 0.2766 |
| Shaw et al, 2005 [12]  | 0–1 year & foetuses with CHD | Age matched          | Conotruncal heart cases confirmed by Echocardiography                               | PB                | America         | Caucasian | DIRECT SEQUENCING | 69      | 180      | 16 | 153  | 0.6836 |
| Zhu et al, 2006 [13]   | 6.2 yrs                      | 8.4 yrs              | Confirmed CHD by Echocardiography                                                    | PB                | China           | Asian     | PCR-RFLP          | 3       | 22       | 12 | 22   | 0.2766 |
| Zhu et al, 2006 [13]   | 6.2 yrs                      | 8.4 yrs              | Confirmed CHD by Echocardiography                                                    | PB                | China           | Asian     | PCR-RFLP          | 4       | 22       | 15 | 34   | 0.2766 |
| van Beynum et al, 2006 [4] | 3.4 yrs                      | 9.4 yrs              | Echocardiography excluding NTD, cleft palate/ lip, detected genetic abnormalities, known syndromes, and VACTERL association | PB                | Caucasian       | Caucasian | PCR-RFLP          | 79      | 98       | 20 | 165  | 0.1842 |
| Study                  | Age Group/ Mean age of cases | Mean age of controls | Diagnostic criteria                                                                                                                                                                                                 | Source of controls | Country/ Region | Ethnicity | Genotyping Method | Cases | Controls | NOS | HWE  |
|-----------------------|------------------------------|----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|-----------------|-----------|-------------------|-------|----------|-----|-------|
| Galdieri et al., 2007 [14] | 0–11 yrs                    | -                    | Isolated cardiopathies (not associated with genetic syndromes or other malformations) confirmed by echocardiogram or cardiac catheterization                                                                 | HB                 | Brazil          | Caucasian | DIRECT SEQUENCING | 30    | 21       | 7   | 58    | 18  | 14       | 6   | 38     | 9   | 0.2631 |
| van Driel et al., 2008 [15] | 16.8 months                 | 16.7 months          | Confirmed CHD by echocardiography and/or cardiac catheterization and/or surgery                                                                                                                                       | PB                 | European        | Caucasian | Real time PCR, RFLP | 99    | 103      | 27 | 229   | 119 | 107      | 25  | 251    | 9   | 0.8951 |
| Xu et al., 2010 [16]     | 6.50 yrs                     | 6.69 yrs             | Non-syndromic CHD cases confirmed by echocardiography                                                                                                                                                            | HB                 | China           | Asian     |                  | 162   | 244      | 96 | 502   | 151 | 261      | 115 | 527    | 0.9115 |
| Kuehl et al., 2010 [17]  | Infants before one year of age | Age matched          | Confirmed CHD by echocardiography and/or cardiac catheterization and/or surgery                                                                                                                                       | PB                 | America         | Caucasian | DIRECT SEQUENCING | 12    | 33       | 10 | 55    | 134 | 134      | 32  | 300     | 7   | 0.8611 |
| Oberman-Borst et al., 2011 [18] | 17 months                  | 17.3 months          | Confirmed CHD by echocardiography and/or cardiac catheterization and/or surgery                                                                                                                                       | PB                 | Netherlands     | Caucasian | DIRECT SEQUENCING | 64    | 66       | 9  | 139   | 92  | 76        | 15  | 183     | 8   | 0.9    |
| Kotby et al., 2012 [19]  | 31.5 months                 | 32.7 months          | Conotruncal heart defects excluding syndrome CHD                                                                                                                                                                     | PB                 | Egypt           | Caucasian | PCR-RFLP       | 12    | 14       | 4  | 30    | 20  | 8         | 2   | 30      | 8   | 0.3613 |
| Study                        | Age Group/ Mean age of controls | Mean age of cases | Diagnostic criteria                                                                 | Source of controls | Country/ Region | Ethnicity | Genotyping Method | Cases CC | Cases CT | Cases TT | Cases Total | Controls CC | Controls CT | Controls TT | Controls Total | NOS | HWE       |
|-----------------------------|---------------------------------|-------------------|--------------------------------------------------------------------------------------|--------------------|-----------------|-----------|-------------------|----------|----------|----------|-------------|-----------|-----------|------------|-----------------|-----|---------|
| Gong et al, 2012 [20]       | 2.27 yrs 1.58 yrs               | 2.27 yrs          | Non-syndromic CHD cases confirmed by echocardiography and/or surgery                  | HB                 | Chinese Han population | Asian     | MALDI-ToF–MS       | 45       | 123      | 76       | 244         | 43        | 72        | 21         | 136             | 8   | 0.3088   |
| El-Abd et al, 2012 [21]     | Neonates                        | Neonates          | Confirmed CHD except congenital heart disease associated with chromosomal anomalies and genetic syndromes, premature infants (<37 weeks gestation) and maternal diabetes, malabsorption, wasting syndromes, or any condition associated with folate deficiency | HB                 | Egypt            | Caucasian | PCR–RFLP          | 7        | 12       | 7        | 26          | 13        | 5         | 0          | 18              | 9   | 0.4938   |
| Wang et al, 2013 [22]       | -                               | -                 | Confirmed CHD by echocardiography                                                     | HB                 | China           | Asian     | SNaPShot genotyping, sequencing | 59       | 76       | 25       | 160         | 53        | 100       | 35         | 188             | 9   | 0.3124   |
| Kocakap et al, 2014 [23]    | 3.7 yrs 8.7 yrs                  | 8.7 yrs           | Patients with echocardiographically proven conotruncal heart defect                   | HB                 | Turkey          | Caucasian | HRM, PCR–RFLP, Sequencing | 40       | 33       | 2        | 75          | 43        | 44        | 8          | 95              | 9   | 0.4841   |
## Table 3 (continued)

| Study                          | Age Group/ Mean age of cases | Mean age of controls | Diagnostic criteria                                                                 | Source of controls | Country/ Region | Ethnicity | Genotyping Method     | Cases          | Controls         | NOS | HWE  |
|-------------------------------|------------------------------|----------------------|-------------------------------------------------------------------------------|-------------------|----------------|-----------|----------------------|----------------|------------------|-----|------|
| Chao et al., 2014 [24]        |                              | 46.7 yrs 50.9 yrs    | Patients undergoing PDA ligation except patients diagnosed with diseases due to chromosomal defect or those born prematurely | HB                | Taiwan         | Asian     | PCR–RFLP            | 10 5 2 17      | 19 12 3 34       | 8   | 0.5863|
| Mohamad et al., 2014 [8]      | Paediatric cases >2.1 years | 7.63 yrs             | Non-syndromic CHD patients confirmed by echocardiography                      | PB                | Malaysians     | Asian     | PCR–RFLP            | 118 32 0 150   | 131 19 0 150      | 7   | 0.4076|
| Sahiner et al., 2014 [25]     | 7.63 yrs                     | -                    | Non-syndromic CHD patients confirmed by echocardiography                      | HB                | Turkey         | Caucasian | PCR–RFLP            | 69 53 14 136   | 47 39 7 93        | 9   | 0.7791|
| Li et al., 2015 [26]          | -                            | -                    | Clinically confirmed CHD patients by echocardiography                        | HB                | China          | Asian     | DIRECT SEQUENCING    | 31 78 41 150   | 59 66 25 150      | 9   | 0.3756|
| Shi et al., 2015 [27]         | -                            | -                    | Clinically confirmed CHD patients by echocardiography                        | PB                | China          | Asian     | PCR–RFLP            | 55 68 30 153   | 70 101 45 216     | 8   | 0.4437|
| Wang et al., 2016 [28]        | 1.46 yrs 3.08 yrs            | Non-syndromic CHD patients confirmed by echocardiogram or cardiac catheterization | HB                | Chinese Han population | Asian     | Taq-Man allelic discrimination assay | 14 73 60 147   | 49 84 35 168      | 9   | 0.9278|
Table 3 (continued)

| Study               | Age Group/ Mean age of cases | Mean age of controls | Diagnostic criteria                                                                 | Source of controls | Country/ Region | Ethnicity | Genotyping Method | Cases | Controls | NOS | HWE  |
|---------------------|-------------------------------|----------------------|-------------------------------------------------------------------------------------|--------------------|-----------------|-----------|-------------------|-------|----------|-----|------|
| Noori et al, 2017   | 4.2 yrs                       | 4.9 yrs              | confirmed CHD patients by echocardiography, cardiac catheterization and surgical procedures | HB Iran Asian     | Tetra-ARMS PCR  | 95 51 7 153     | 100 46 1 147 | 9 0.0781            |
| Wang et al, 2018    | -                             | -                    | Conotruncal heart defects CHD patients by echocardiography                             | HB China Asian    | DIRECT SEQUENCING | 8 48 36 92    | 70 117 50 237 | 7 0.9316            |
| Present study 2020 | 23.24 months                  | 59.26 months         | Non-syndromic CHD patients confirmed by echocardiography and surgical procedures     | HB Indian Asian   | PCR–RFLP        | 44 4 2 50      | 90 9 1 100 | 8 0.1796            |
GA genotypes; however we did not observe any AA genotype in controls. In general there was higher frequency of risk allele 'A' in CHD patients (0.23) in comparison to controls (0.05). The distribution of observed MS genotypes in CHD patients were 60%, 36% & 4% for AA, AG and GG genotypes respectively. In control group the distribution was as follows: 73% for AA, 26% for AG and 1% for GG genotype. The CHD patients were showing higher frequency of risk allele ‘G’ (0.22) than controls (0.14).

In order to investigate the possible association of these three polymorphisms with susceptibility of CHD, ORs with 95% confidentiality intervals was calculated for different genetic models which are presented in Table 6.

For both \textit{MTHFR} C677T and \textit{MS} A2756G polymorphisms, we observed that even though the values calculated for ORs under different models were above 1, but none of the values reached statistical significance level ($p > 0.05$). The present study proclaimed lack of association of \textit{MTHFR} C677T and \textit{MS} A2756G gene polymorphism with the risk of CHD in our population. Furthermore, the GA vs GG genotype depicted a strong significant association of \textit{MTHFR} G1793A gene polymorphism. The G vs A frequency showed that the allele ‘A’ is adding a significant risk of approximately 5.7 folds in the development of CHD in the studied population. Distribution of \textit{MTHFR} haplotypes in cases & controls and their association towards CHD susceptibility is depicted in Table 7.

The frequency of C-G haplotype was higher in both cases and controls (0.690 & 0.895 respectively). There was complete absence of T-A haplotype in both study groups. The haplotype combination C-A was significantly associated with CHD risk ($\text{OR} = 5.67 \ [2.58–12.48]$, $p = 2.71 \cdot 10^{-06}$) and C-G was significantly involved in protection against CHD development ($\text{OR} = 0.26 \ [0.14–0.48]$, $p = 1.00 \cdot 10^{-05}$) in the population under reference. By analysing LD scores in two study groups it was observed that the \textit{MTHFR} variants were in complete

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| Table 4 | Prevalence of CHD phenotypes in present study |
|-----------------------------------|---------------------------------|
| Type of CHD | No. of Cases ($N = 50$) | Percentage (%) |
|-----------------|-----------------|----------------|
| Ventricular septal defect (VSD) | 17 | 34% |
| Atrial septal defect (ASD) | 13 | 26% |
| Tetralogy of fallot (TOF) | 7 | 26% |
| Patent ductus arteriosus (PDA) | 4 | 8% |
| Endocardial cushion defect | 3 | 6% |
| ASD with PDA | 2 | 4% |
| VSD with peripheral arterial hypertension | 2 | 4% |
| VSD with AV-canal defect | 1 | 2% |
| Endocardial cushion defect along with dextrocardia | 1 | 2% |

| 
| Table 5 | Showing genotypic and allelic distribution of selected gene polymorphisms among cases and controls |
|-----------------------------------|---------------------------------|
| Category | Genotypes/Alleles (%) | $\chi^2$ | $p$-value |
|-----------------------------------|---------------------------------|
| 
| \textit{MTHFR} (C677T) polymorphism |
| CC (Wild) | CT (Hetero) | TT (Risk) | C (Wild) | T (Risk) |
| CHD Cases ($n = 50$) | 44 (88%) | 4 (8%) | 2 (4%) | 0.92 | 0.08 | 10.42 | 0.001* |
| Controls ($n = 100$) | 90 (90%) | 9 (9%) | 1 (1%) | 0.95 | 0.05 | 1.8 | 0.18 |

| \textit{MTHFR} (G1793A) polymorphism |
| GG (Wild) | GA (Hetero) | AA (Risk) | G (Wild) | A (Risk) |
| CHD Cases ($n = 50$) | 29 (58%) | 19 (38%) | 2 (4%) | 0.77 | 0.23 | 0.27 | 0.61 |
| Controls ($n = 100$) | 90 (90%) | 10 (10%) | 0 | 0.95 | 0.05 | 0.28 | 0.60 |

| \textit{MS} (A2756G) gene polymorphism |
| AA (Wild) | AG (Hetero) | GG (Risk) | A (Wild) | G (Risk) |
| CHD Cases ($n = 50$) | 30 (60%) | 18 (36%) | 2 (4%) | 0.78 | 0.22 | 0.12 | 0.73 |
| Controls ($n = 100$) | 73 (73%) | 26 (26%) | 1 (1%) | 0.86 | 0.14 | 0.64 | 0.43 |
LD in both patients \((D' = 0.999, r^2 = 0.026)\) and controls \((D' = 1, r^2 = 0.003)\).

### Meta-analysis

We found 26 eligible studies having 3450 cases and 4447 controls with reference to \(MTHFR\) C677T polymorphism and 6 studies with 697 cases and 777 controls concerning \(MS\) A2756G polymorphism. The main study characteristics are summarized in Table 3. The study selection process has been depicted in PRISMA diagram (Fig. 4). By pooling all the studies, it was found that there is statistically significant association between \(MTHFR\) C677T polymorphism and congenital heart defects under all applied genetic models (Dominant model: \(OR = 1.38, 95\% CI: 1.14-1.69\); recessive model: \(OR = 1.49, 95\% CI: 1.83-1.87\); allele model: \(OR = 1.33, 95\% CI: 1.14-1.55\)) as shown in Table 8 and Fig. 5, 6, and 7. When we stratified the studies according to ethnicity, a significant association was observed between this locus and CHD only in Asian populations (Dominant model: \(OR = 1.50, 95\% CI: 1.12-2.01\); recessive model: \(OR = 1.67, 95\% CI: 1.21-2.31\); allele model: \(OR = 1.42, 95\% CI: 1.15-1.76\), but not in Caucasian populations (dominant model: \(OR = 1.24, 95\% CI: 0.95-1.62\); Recessive model: \(OR = 1.27, 95\% CI: 0.99-1.63\); allele model: \(OR = 1.21, 95\% CI: 0.97-1.50\)) as given in Table 8.

However, it was observed that Caucasian population was also showing association but it did not reach statistical significance. For \(MS\) polymorphism, none of the applied genetic models found association with CHD in overall population or even after subgrouping (Table 9 and Fig. 8). Sensitivity analysis for both \(MTHFR\) and \(MS\) revealed that there is no change in the pooled ORs by omitting individual studies (Fig. 9 and 10). The publication bias was also estimated by using funnel plot for log-odds ratio against the reciprocal of its standard error (Fig. 11 and 12). Further Egger regression asymmetry test was also used to evaluate publication bias (Table 9). No publication bias was observed in the present meta-analysis.

Meta-analysis could not be performed for \(MTHFR\) G1793A gene polymorphism as we were able to find only one study other than the study under reference. Further, it was also observed that Caucasian population was also showing association but it did not reach statistical significance. For \(MS\) polymorphism, none of the applied genetic models found association with CHD in overall population or even after subgrouping (Table 9 and Fig. 8).

### Trial Sequential Analysis (TSA)

Trial sequential analysis was performed to calculate the requisite sample size for the meta-analysis of \(MTHFR\) C677T gene polymorphism. It revealed that
sufficient number of studies have been included in the meta-analysis of this polymorphism. The results of TSA were in accordance with the findings of the conventional meta-analysis and revealed that C677T polymorphism was significantly associated with the risk of CHD (Fig. 13). For MS A2756G polymorphism, TSA could not be performed owing to very little information of sample size which revealed that there is need of more replicas of case control studies to reach the conclusive remarks on role of said polymorphism in conferring risk of CHD. Similarly for MTHFR G1793A gene polymorphism, TSA could not be performed as only two studies were available for meta-analysis.

**Discussion**

The folate-homocysteine metabolic pathway performs a paramount role in neural tube formation and cardiac development during embryogenesis. Low folate and high homocysteine levels are a closely related with the manifestation of congenital heart defects, which indicates that single nucleotide polymorphisms (SNPs) in the genes controlling this pathway may be the genetic risk factors for these disorders [34]. Therefore, we performed a case-control association study and an updated meta-analysis along with TSA to investigate the association of MTHFR and MS gene polymorphisms with risk of CHD. We did not find a significant association of MTHFR C677T and MS A2756G polymorphism with risk of CHD in our studied population. The results were consistent with studies done by various workers [5, 7, 35–38]. Regarding MTHFR G1793A polymorphism in link with CHD risk we found significant association under co-dominant, dominant and allelic model in present study. The genotypic frequencies reported in the present study were almost compatible with frequencies as reported by Toganel and co-workers and the investigators also observed a
Table 8  Overall meta-analysis and subgroup analysis by ethnicity for MTHFR C677T polymorphism

| Genetic Model                              | Number of studies | Test of association | Heterogeneity | Egger's test |
|--------------------------------------------|-------------------|---------------------|---------------|--------------|
|                                            |                   | OR                  | 95% CI        | p-value      | Model | p-value | I^2 | p-value |
| Overall                                    |                   |                     |               |              |        |         |     |         |
| Allele contrast (T vs. C)                  | 26                | 1.33                | 1.14–1.55     | 0.0002       | Random | 0.0001  | 0.7554 | 0.0259  |
| Recessive model (TT vs. TC + CC)           | 25^a              | 1.49                | 1.83–1.87     | 0.0007       | Random | 0.0001  | 0.5828 | 0.1945  |
| Dominant model (TT +TC vs. CC)             | 26                | 1.38                | 1.14–1.69     | 0.001        | Random | 0.0001  | 0.696  | 0.0068  |
| Homozygous model (TT vs CC)                | 25^a              | 1.75                | 1.26–2.44     | 0.001        | Random | 0.0001  | 0.7286 | 0.0699  |
| Heterozygous model (TT vs CT)              | 25^a              | 1.34                | 1.11–1.60     | 0.002        | Random | 0.02    | 0.5157 | 0.6033  |
| Caucasians                                 |                   |                     |               |              |        |         |     |         |
| Allele contrast (T vs. C)                  | 11                | 1.21                | 0.97–1.50     | 0.1          | Random | 0.0006  | 0.6755 | 0.1529  |
| Recessive model (TT vs. TC + CC)           | 11                | 1.27                | 0.99–1.63     | 0.06         | Fixed  | 0.1662  | 0.2933 | 0.8658  |
| Dominant model (TT +TC vs. CC)             | 11                | 1.24                | 0.95–1.62     | 0.1          | Random | 0.003   | 0.6234 | 0.0657  |
| Homozygous model (TT vs CC)                | 11                | 1.37                | 0.91–2.07     | 0.1          | Random | 0.0237  | 0.5157 | 0.6033  |
| Heterozygous model (TT vs CT)              | 11                | 1.78                | 0.91–1.53     | 0.2          | Fixed  | 0.5288  | 0  | 0.8349  |
| Asians                                     |                   |                     |               |              |        |         |     |         |
| Allele contrast (T vs. C)                  | 15                | 1.42                | 1.15–1.76     | 0.001        | Random | 0.0001  | 0.7988 | 0.0765  |
| Recessive model (TT vs. TC + CC)           | 14^a              | 1.67                | 1.21–2.31     | 0.002        | Random | 0.0001  | 0.6958 | 0.1205  |
| Dominant model (TT +TC vs. CC)             | 15                | 1.50                | 1.12–2.01     | 0.02         | Random | 0.0001  | 0.7438 | 0.0599  |
| Homozygous model (TT vs CC)                | 14^a              | 2.12                | 1.30–3.47     | 0.003        | Random | 0.0001  | 0.8067 | 0.08    |
| Heterozygous model (TT vs CT)              | 14^a              | 1.46                | 1.13–1.89     | 0.003        | Random | 0.03    | 0.4697 | 0.0834  |

^a In one of the study, TT genotype is completely absent in one of the study group

**Fig. 5**  Pooled OR (Dominant model) and 95% CI for individual studies and pooled data for the association between the polymorphism C677T and congenital heart disease (CHD) in the overall population
strong significant association this SNP with susceptibility of CHD [AA + GA vs GG: OR = 4.18; 95% CI (1.25-13.98), \( p = 0.02 \)] in a Romanian population whereas antithetical findings were reported in Chinese population [39, 40]. Xu and co-workers found that the variant genotypes of \( MTHFR \) G1793A polymorphism were significantly associated with a decreased risk of CHD, especially in patients with isolated peri-membranous VSD [40]. The correlation between the \( MTHFR \) G1793A gene polymorphism and the CHD risk has not been extensively studied so far. To the best of our knowledge there is no previous report from India and we are the first to analyse G1793A variation of \( MTHFR \) gene from North India. The present study is first of its kind concentrating on the effect of \( MTHFR \) (C677T and G1793A) haplotypes with vulnerability of CHD. The haplotype C-A was conferring nearly 5.7-fold disease risk and C-G haplotype was giving a shielding outcome of approximately 3.8-fold (1/0.26). Based on measure of LD, the two \( MTHFR \) SNPs were in complete LD in both CHD cases and controls. The possible limitations of the present study may be the enrolment of study samples from single region of UT J&K and lack of homocysteine measurements in the study subjects. Besides these limitations and to the best of our knowledge, the study under reference here is the first attempt that evaluates the association of \( MTHFR \) and \( MS \) gene polymorphisms in CHD.

Genetic association studies have been a powerful approach for identifying susceptibility genes for common diseases but it has been experienced that most of the initial positive associations were not reproduced in the subsequent replication studies because of small sample size or false-positive reports [41, 42]. Meta-analysis solves this problem as it increases the statistical power to detect gene–disease associations by combining results from the original and subsequent replication studies [42]. Similarly, when we conducted case–control association, we did not observe significant association of \( MTHFR \) C677T with risk of CHD, as it was a pilot study and carried on limited number of samples. But after performing meta-analysis, the results suggested a positive association of \( MTHFR \) C677T with the risk of CHD.

### Table 1

| Study                     | Experimental Events | Control Events | Odds Ratio | OR    | 95%-CI | W(fixed) | W(random) |
|---------------------------|---------------------|----------------|------------|-------|--------|----------|-----------|
| Junker et al., 2001       | 21                  | 114            | 21         | 2.23  | [1.16; 4.27] | 4.2%     | 4.9%      |
| Lee et al., 2005          | 14                  | 213            | 13         | 0.98  | [0.45; 2.15] | 2.9%     | 4.2%      |
| Li et al., 2005           | 58                  | 183            | 24         | 1.53  | [0.88; 2.65] | 5.8%     | 5.6%      |
| Shaw et al., 2005         | 16                  | 153            | 52         | 0.86  | [0.47; 1.55] | 5.0%     | 5.3%      |
| Zhu et al., 2006          | 12                  | 22             | 24         | 3.95  | [1.52; 10.27] | 1.9%     | 3.4%      |
| Zhu et al., 2006_1        | 15                  | 34             | 24         | 2.60  | [1.15; 5.88] | 2.7%     | 4.0%      |
| van Beynum et al., 2006   | 20                  | 165            | 18         | 1.55  | [0.79; 3.03] | 3.9%     | 4.8%      |
| Galdieri et al., 2007     | 7                   | 58             | 6           | 0.73  | [0.23; 2.37] | 1.3%     | 2.8%      |
| van Driel et al., 2008    | 27                  | 229            | 25          | 1.21  | [0.68; 2.15] | 5.3%     | 5.4%      |
| Xu et al., 2010           | 96                  | 502            | 115         | 0.85  | [0.63; 1.15] | 19.2%    | 7.2%      |
| Kuehle et al., 2010       | 10                  | 55             | 32          | 1.86  | [0.86; 4.05] | 2.9%     | 4.2%      |
| Oberman-Borst et al., 2011| 9                   | 139            | 15          | 0.78  | [0.33; 1.83] | 2.4%     | 3.8%      |
| Kolby et al., 2012         | 4                    | 30             | 2           | 2.15  | [0.36; 12.76] | 0.6%     | 1.4%      |
| Gong et al., 2012         | 76                   | 244            | 21          | 2.48  | [1.45; 4.24] | 6.1%     | 5.7%      |
| El-Abd et al., 2012       | 7                    | 26             | 0           | 14.23 | [0.78; 267.18] | 0.2%     | 0.8%      |
| Wang et al., 2013          | 25                   | 160            | 35          | 0.81  | [0.46; 1.42] | 5.6%     | 5.5%      |
| Kocakap et al., 2014       | 2                    | 75             | 8           | 0.30  | [0.06; 1.46] | 0.7%     | 1.7%      |
| Chao et al., 2014          | 2                    | 17             | 3           | 1.38  | [0.21; 9.14] | 0.5%     | 1.3%      |
| Mohamad et al., 2014       | 0                    | 150            | 0           | 0.00  | [0.00; 1.00] | 0.0%     | 0.0%      |
| Sahiner et al., 2014       | 14                   | 136            | 7           | 1.41  | [0.55; 3.64] | 2.0%     | 3.4%      |
| Li et al., 2015            | 41                   | 150            | 25          | 1.88  | [1.07; 3.29] | 5.7%     | 5.5%      |
| Shi et al., 2015           | 30                   | 153            | 45          | 0.93  | [0.55; 1.55] | 6.6%     | 5.8%      |
| Wang et al., 2016          | 60                   | 147            | 35          | 2.62  | [1.59; 4.31] | 7.2%     | 5.9%      |
| Noon et al., 2017          | 7                    | 153             | 1          | 7.00  | [0.85; 57.61] | 0.4%     | 1.0%      |
| Wang et al., 2018          | 36                   | 92             | 50           | 2.40  | [1.43; 4.05] | 6.5%     | 5.8%      |
| Present study 2020         | 2                    | 50             | 1           | 4.12  | [0.39; 46.83] | 0.3%     | 0.8%      |

**Fig. 6** Pooled OR (Recessive model) and 95% CI for individual studies and pooled data for the association between the polymorphism C677T and congenital heart disease (CHD) in the overall population.
Table 9  Overall meta-analysis and subgroup analysis by ethnicity for MS A2756G polymorphism

| Genetic Model                  | Number of studies | Test of association | Heterogeneity | Egger's test |
|--------------------------------|-------------------|---------------------|---------------|--------------|
|                                |                   | OR                  | 95% CI        | p-value      |
|                                |                   | Fixed               | Random        | p-value      |
|                                |                   | I^2                 | p-value       | I^2          |
| Overall                        |                   |                     |               |              |
| Allele contrast (G vs. A)      | 6                 | 1.05                | 0.88–1.26     | 0.6          | Fixed        | 0.3          | 0.1993 | 0.4631 |
| Recessive model (GG vs. AG+AA) | 6                 | 1.11                | 0.47–2.46     | 0.8          | Random       | 0.07         | 0.5136 | 0.0517 |
| Dominant model (GG+AG vs. AA)  | 6                 | 1.08                | 0.86–1.35     | 0.5          | Fixed        | 0.6          | 0       | 0.7422 |
| Homozygous model (GG vs AA)    | 6                 | 0.95                | 0.57–1.56     | 0.8          | Fixed        | 0.1          | 0.4122 | 0.4344 |
| Heterozygous model (GG vs AG)  | 6                 | 1.10                | 0.45–2.72     | 0.8          | Random       | 0.06         | 0.5204 | 0.5685 |
| Caucasians                     |                   |                     |               |              |
| Allele contrast (G vs. A)      | 3                 | 0.95                | 0.75–1.19     | 0.6          | Fixed        | 0.5          | 0       | 0.9501 |
| Recessive model (GG vs. AG+AA) | 3                 | 0.86                | 0.30–2.47     | 0.8          | Random       | 0.03         | 0.7067 | 0.9516 |
| Dominant model (GG+AG vs. AA)  | 3                 | 0.96                | 0.71–1.31     | 0.8          | Fixed        | 0.92         | 0       | 0.0379 |
| Homozygous model (GG vs AA)    | 3                 | 0.84                | 0.34–2.06     | 0.7          | Random       | 0.1          | 0.5605 | 0.9324 |
| Heterozygous model (GG vs AG)  | 3                 | 0.87                | 0.26–2.91     | 0.8          | Random       | 0.02         | 0.7476 | 0.9915 |
| Asians                         |                   |                     |               |              |
| Allele contrast (G vs. A)      | 3                 | 1.25                | 0.91–1.69     | 0.1          | Fixed        | 0.3          | 0.2455 | 0.6974 |
| Recessive model (GG vs. AG+AA) | 3                 | 2.26                | 0.51–9.94     | 0.3          | Fixed        | 0.4          | 0.0104 | 0.5599 |
| Dominant model (GG+AG vs. AA)  | 3                 | 1.24                | 0.48–3.07     | 0.21         | Fixed        | 0.3          | 0.0785 | 0.5501 |
| Homozygous model (GG vs AA)    | 3                 | 2.42                | 0.55–10.69    | 0.2          | Fixed        | 0.3          | 0.1005 | 0.377 |
| Heterozygous model (GG vs AG)  | 3                 | 1.95                | 0.43–8.78     | 0.4          | Fixed        | 0.5          | 0       | 0.4763 |
The results of the overall analysis depicted an increased risk of CHD with the presence of MTHFR 677 T-allele in fetus. The putative risk allele-677 T had a 1.33 folds increased risk of CHD against the C-allele. From the subgroup analysis, the increased risk of the T-allele was widely detected in Asians but not in Caucasians. Our results are compatible with the previous Meta analyses that investigated the association of the MTHFR C677T polymorphism in CHD [34, 43]. Further, this association revealed through conventional meta-analysis has also been confirmed by performing Trial Sequential Analysis. Lack of association was reported for MS A2756G both
in pooled and in sub-grouped meta-analysis and the findings are consistent with study done by Cai and co-workers [44]. The findings of MS polymorphism needs to be further investigated as there are not enough studies on association of this polymorphism with risk of CHD and during our search we also found only six eligible studies and TSA has not been performed in lieu of lack of sufficient number of studies. Further, we were not able to perform meta-analysis for MTHFR G1793A polymorphism as to best of our efforts; we found only a few case–control studies which were not sufficient for performing meta-analysis.
Fig. 12 Funnel plot of the MS/MTR A2756G polymorphism and susceptibility to CHD (Dominant model) in the overall population

Fig. 13 Trial Sequential Analysis (TSA) of the studies included in the meta analysis of MTHFR C677T gene polymorphism with CHD
Conclusion
In conclusion, the results of meta-analysis and TSA support the role of MTHFR C677T gene polymorphism as susceptibility factor for Congenital Heart Defects. For MTHFR G1793A and MS A2756G gene polymorphisms, there is need to perform large number of homogenous studies to evaluate these crude results further.

Abbreviations
CHD: Congenital Heart Defects; MTHFR: Methylenetetrahydrofolate reductase; MTR: 5-Methyltetrahydrofolate-Homocysteine Methytransferase; TSA: Trial sequential analysis; PCR: Polymerase Chain Reaction; RFLP: Restriction Fragment Length Polymorphism; PCR–RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; CVD: Cardiovascular Disease; S, 10-MTHF; S, 10-Methylenetetrahydrofolate; 5-MTHF: 5-Methyltetrafolate; MS: Methionine synthase; HWE: Hardy–Weinberg equilibrium; OR: Odd Ratio; CI: Confidence Interval; SPSS: Statistical Package for Social Sciences; NOS: Newcastle–Ottawa Scale; I^2: I-square; VSD: Ventricular septal defect; ASD: Atrial septal defect; TOF: Tetralogy of fallot; PDA: Patent ductus arteriosus; VSD-PAH: Ventricular septal defect with pulmonary arterial hypertension; LD: Linkage Disequilibrium; PRISMA: Preferred Reporting Items for Systematic Reviews and Meta-Analyses; SNPs: Single nucleotide polymorphisms; AV canal defect: Atioventricular canal defect.

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Authors’ contributions
JKR and A carried out the sampling and lab work for the conduct of research under reference. VD: carried out data interpretation and manuscript writing. RKP and PK: participated in the study design and conceptualization. SS carried out the clinical diagnosis and recruitment of patients. All the authors undertake to declare that they have read the complete manuscript before submission to the journal. The authors(s) read and approved the final manuscript.

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Availability of data and materials
The data and the material used in the research work under reference can be made available upon reasonable request from corresponding author.

Declarations
Ethics approval and consent to participate
Ethical approval for the conduct of present research work was taken from the Institutional Ethical Committee, University of Jammu. All the methods were carried out in accordance with relevant guidelines and regulations. Data collection and blood sampling were done after getting prior informed consent from mother/guardian of the subject(s).

Consent for publication
Not applicable.

Competing interests
The authors declare that they do not have any conflict of interest.

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