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REVIEW ARTICLE

Congenital heart defects among Down's syndrome cases: an updated review from basic research to an emerging diagnostics technology and genetic counselling

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Abstract. Congenital heart defects (CHD) affect 50% of Down's syndrome (DS) cases. This review focusses on the pathogenic molecular mechanism leading to the formation of DS-associated CHD along with the advancement of the emerging diagnostic techniques available for such patients in past few decades. We have shed light on the causative genes of DS-associated CHD that are located either on chromosome 21 or outside chromosome 21. Along with locus-specific mutation, numerous SNP and CNV, miRNA, use of maternal folic acid during pregnancy and signalling pathways are also reported to contribute to the formation of CHD in patients with DS. With the help of both these our understanding of pathogenic mechanism causing CHD in DS cases along with the availability of emerging technologies has facilitated a novel discovery that has ultimately provided a better treatment and management for such cases. Accurate diagnosis and treatment are now available with the introduction of CNV detection and NGS based approaches such as WES, WGS, target sequencing and sequencing of foetal cell-free DNA by the medical geneticist and cardiologist have now allowed further identification of familial recurrence risk and relatives who are at risk through genetic counselling, thereby providing reproductive options and improving proper care of DS-associated CHD. Further, gene-editing studies explore novel pathogenic mechanisms and signalling pathways in DS-associated CHD.

Keywords. Down's syndrome; congenital heart defects; atrioventricular septal defects; next-generation sequencing; genetic counselling.

Introduction of CHD

Congenital heart defects (CHD), a common congenital anomaly that accounts for ∼10−12 per 1000 live birth (Weijerman *et al.* 2008). Mostly, the CHD is not diagnosed in early infancy or childhood because the actual prevalence remains undetermined. CHD is multifactorial in nature and ∼20–30% of CHD cases are caused due to genetic and environmental factors. CHD along with other clinical conditions forms a part of various syndromes, where the percentage of CHD in chromosomal aneuploidy is 8–10%, in single-gene disorders it is 3–5%, in copy number variation it is 3–25%, in an isolated form of CHD is 3–10%, in *de novo* autosomal dominant disorders is 8% and in inherited autosomal recessive disorders are 2% (Freeman *et al.* 2008; Morris *et al.* 2014; Stoll *et al.* 2015). Therefore, genetic counselling by a medical geneticist and cardiologist in combination have become an integral part in determining the genetic cause of CHD to evaluate the recurrent risk of siblings and relatives of patients with CHD, and to provide a more precise prognosis and better management for CHD.

DS-associated CHD

DS is caused due to several reasons including meiotic nondisjunction (accounts for 88% of cases), Robertsonian translocation (4.5%), mosaicism (2–3%) and duplication (a rare event) (Hassold and Hunt 2001; Asim *et al.* 2015). DS is also associated with various other clinical conditions such as Alzheimer's disease (AD), cancer, speech, learning and memory defects, CHD, Hirschsprung's disease (HD) and leukaemia (Hassold and Hunt 2001; Epstein 2001; Antonarakis *et al.* 2004; Ermak *et al.* 2006; Miko 2008; Asim *et al.* 2015). The prevalence of CHD in DS is estimated to be

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Table 1. Details of syndromic, nonsyndromic and familial AVSD.

Syndromic AVSD (accounts for 64% of the total cases)			
	Description	Genes involved	Pathway associate/animal studies
Syndromic AVSD and chromosomal anomalies			
DS	CHD in DS accounts for 40–63% of DS cases	• Genes present in DSCR, COL6A1, COL6A2, DSCAM and DSCR1 • Genes present on different chromosome: *CRELD1*, *FBLN2*, *FRZB*, and *GATA5*	Mouse models have confirmed the connection of the Shh signalling pathway DS (Asim *et al.* 2015)
Deletion 3p25	Cardiac defects are present in ∼33% patients having deletion 3p25 (Phipps *et al.* 1994; Drumheller *et al.* 1996; Green *et al.* 2000)	*CRELD1* gene is located on chromosome 3p25	CRELD1 mutations affects SHF Hh signalling (Phipps *et al.* 1994; Drumheller *et al.* 1996; Green *et al.* 2000)
Deletion 8p23	• Deletion of the p arm chromosome 8 • ∼67 % of patients have cardiac defects • AVSD accounts for 40% of these cases • Other sub-types of CHD include pulmonary valve stenosis and TOF are present	*GATA4* is the causative gene which maps to the 8p23.1 region and is expressed in the developing heart	GATA4 interacts with transcriptional factors involved in SHH signalling
Syndromic AVSD and monogenic disorder			
Ciliopathies	Ciliopathies with AVSD can be divided in the following way: • Syndromes including polydactyly - Results in ciliary dysfunction due to atypical processing of Hh proteins. For example: Bardet-Biedl, Ellis-van Creveld, oral-facial digital syndromes and Smith–Lemli–Opitz • Syndromes not including polydactyly: VACTERL association and ACD		
Syndromes with polydactyly			
Ellis-van Creveld syndrome	• Autosomal recessive disorder • Short-limb dwarfism, short ribs, postaxial polydactyly of hands and feet, ectodermal defects and CHD • ∼66% has cardiac defects	Mutations in causative genes, namely *EVC*, *EVC2*, *WDR35* and *DYNC2LI1*	*EVC* and *EVC2* genes leads to activation of transcription HH signalling (Ruiz-Perez *et al.* 2007; Digilio *et al.* 2019; Thomas *et al.* 2012)
Oral-facial-digital syndrome	• Eighteen clinical subtypes • Overlapping clinical features such as deformity of oral cavity, face, and polysyndactyly along with AVSD	WDPCP and TCTN3 are causative genes	SHH signalling
Joubert syndrome	• Deformities present in retina, kidneys, liver, and skeleton. • AVSD is commonly seen	Approximately 30 candidate genes have been identified	
Bardet–Biedl syndrome	• Autosomal recessive disorder • Clinical features include Obesity, genitourinary malformations, retinitis pigmentosa, cognitive impairment, postaxial polydactyly, and CHD	Many genes are involved in ciliary function regulation (Digilio *et al.* 2006)	
Smith–Lemli–Opitz syndrome	• Autosomal recessive syndrome • Clinical features- Growth and developmental delay, cleft lip, toe syndactyly, CHD(AVSD), polydactyly and facial abnormalities • CHD affects 50% SLOS patients	Mutations in *DHCR7* gene causes deficiency of 7-dehydrocholesterol-7 reductase (DHCR7) activity	Variants in DHCR4 disrupts HH signalling pathway (Gurrieri *et al.* 2007).

Table 1 (*contd*)

Syndromic AVSD (accounts for 64% of the total cases)			
	Description	Genes involved	Pathway associate/animal studies
Syndromes without polydactyly			
VACTERL association	• Clinical features are VATERL -Vertebral defects (V) -Anal atresia (A) -CHD (C) -Oesophageal atresia (TE) -Radial and renal dysplasia (R) -Limb anomalies (L) • CHDs in VACTERL is 50%–80% • AVSD, conotruncal and laterality CHD defects are common	• Studies in mice showed disruptive SHH signaling pathway • Causative genes are Ift42, FOXF1 and ZIC3	Disruptive SH signalling pathway (Botto *et al.* 1997)
Alveolar capillary dysplasia	• CHD occurs in 10% • Malformation of pulmonary vessels	Pathogenic variants in FOXF1 gene	FOXF1 gene is an activator of SHH pathway. Mutation disrupts SHH pathway (Laux *et al.* 2013)
RASopathies	• Noonan and related syndromes such as LEOPARD syndrome, cardio-facio-cutaneous syndrome, Costello syndrome and Mazzanti syndrome • CHD is around 65%–85%	Mutation in genes involved in RAS/MAP kinase (MAPK) signalling pathway including PTPN11 and RAF1	RAS/MAP kinase (MAPK) signalling pathway (Tartaglia *et al.* 2010; Aoki *et al.* 2016)
CHARGE syndrome	CHD is present in 80% with AVSD is the prevalent form	Mutations in CHD7 gene (Trip *et al.* 2002)	
Holoprosencephaly	• Characterized by severe congenital forebrain disorder with a broad spectrum of facial anomalies • AVSD is the common sub-type of CHD	Candidate genes are Shh, DKK1, GLI, SIX3, PTCH1, TDGF1, TGIF and ZIC2	Shh pathway gets dysregulated (Wyse *et al.* 1993)
Nonsyndromic atrioventricular canal defects (accounts for 36% of the CHD cases)			
	• Occurs in 1/4th of sporadic cases (Digilio *et al.* 1993) • Autosomal dominant • Incomplete penetrance • Maternal risk factors like genetic and environmental can pose risk for nonsyndromic CHD	Candidate genes are: • CRELD1 (Maslen *et al.* 2006a), PTPN11 (D'Alessandro *et al.* 2016) and NIPBL, CHD7, CEP152, BMPR1a, ZFPM2, MDM4 [100] GATA 4 (Al Turki *et al.* 2014) and NR2F2 (Priest *et al.* 2016). Priest *et al.* reported de novo variants in several genes such as NR1D2, IFT140, MYH6, ADAM17, BBS2, RYR1, CHRD, ZFPM2, PTPRJ, ATE1, NOTCH1, NSD1, VCAN, NOTCH2, SRCAP, KMT2D, and EHMT1(Digilio *et al.* 1993)	• *CRELD1* gene regulator of calcineurin/NFATc1 signalling (Maslen *et al.* 2006b; Bean Lora *et al.* 2011) • GATA4- HH signalling (Alharbi *et al.* 2018)

Table 1 (*contd*)

Syndromic AVSD (accounts for 64% of the total cases)			
	Description	Genes involved	Pathway associate/animal studies
Familial AVSD	• Baltimore Washington Infants Study highlights that around three to five per cent chances of having familial recurrences in cases of nonsyndromic CHDs/AVSD • Recurrence risk accounts for 3.6% in cases of CHD among siblings (Nora *et al.* 1991; Demal *et al.* 2019) • Molecular mechanism of familial AVSD is unexplored • Linkage analysis and NGS based techniques can assess complex trait of recurrence of CHDs • An up-to-date knowledge of the molecular insight of familial AVSD and family history can give substantial clinical result for genetic counselling	• Missense mutations observed in BMPR1A gene (Nora *et al.* 1991) • TBX20 and Tbx2 (Demal *et al.* 2019)	BMPR1A controls the formation of AV cushions through Wnt/ß-catenin signalling (Nora *et al.* 1991)

40 to 63.5%. The sub-types CHD accounts for 45%, 35%, 8%, 7% and 4% for atrioventricular septal defects (AVSD), ventricular defects (VSD), isolated atrial septal defects (ASD), patent ductus arteriosus (PDA) and tetralogy of Fallot (TOF), respectively (Mourato *et al.* 2014; Asim *et al.* 2015; Bermudez *et al.* 2015; Bergstrom *et al.* 2016).

For the past few years, DS-associated CHD is becoming less common in offspring diagnosed with DS. This decline in the number of cases can be attributed because of two reasons: (i) due to miscarriages of DS foetuses; and (ii) lack of availability of better genetic counselling and improvements in antenatal diagnostics. Researchers now know the genome of CHD in DS, still, the underlying cause of CHD in DS remains speculative. Here we summarize a current insight of CHD and DS-associated CHD and have shed some light on possible future research direction in this perspective.

Genetic heterogeneity of AVSD

AVSD accounts for 7.4% of cardiac defects. AVSD is further classified as complete, partial, and intermediate types. Approximately 64% of cases of AVSD are associated with genetics syndrome while 36% of cases account for non-syndromic AVSD (Digilio *et al.* 1998). Following are the classification of syndromic and nonsyndromic associated AVSD (table 1).

(I) Syndromic AVSD and chromosomal anomalies that include: (a) DS (Asim *et al.* 2015); (b) deletion 8p23 (Digilio *et al.* 1998); (c) deletion 3p25 (Phipps *et al.* 1994; Drumheller *et al.* 1996; Green *et al.* 2000).

(II) Syndromic AVSD and monogenic disorder. Ciliopathies include syndromes that includes polydactyly or syndromes that do not include polydactyly. (i) Syndromes including polydactyly: Ellis-van Creveld (Ruiz-Perez *et al.* 2007; Digilio *et al.* 2019; Thomas *et al.* 2012), Smith–Lemli–Opitz (Gurrieri *et al.* 2007), oral-facial digital syndromes (Ferrante *et al.* 2006) and Bardet–Biedl syndrome (Digilio *et al.* 2006). (ii) Syndromes not including polydactyly: alveolar capillary dysplasia (Laux *et al.* 2013) and VACTERL association (Botto *et al.* 1997). (iii) RASopathies (Tartaglia *et al.* 2010; Aoki *et al.* 2016). (iv) CHARGE syndrome (Wyse *et al.* 1993; Trip *et al.* 2002).

Pathogenesis of CHD in DS

Two possible mechanisms reported to date to explain the DS-associated CHD condition. First one is, the ‘gene dosage effect’ hypothesis of DS, that states the increased amount of genes present on human chromosome 21 (Hsa21) can in turn increases gene expression in DS patients (Antonarakis *et al.* 2004; Letourneau *et al.* 2014). Since, the gene-dosage effects lead to overexpression of genes present on chromosome 21 that will also affect the CHD-related genes present on chromosome 21 ultimately causing increased expression of CHD-related genes on chromosome 21 and giving rise to various defects including CHD in DS cases. While the second hypothesis claims that the occurrence of CHD can be due to various locus-specific mutations. The concept of DS critical region also known as ‘DS-CHD critical region’, was first introduced in 1992 by Korenberg *et al*. which was later,

Table 2. List of genes involved in the occurrence of CHD in DS patients present either on chromosome 21 or other chromosomes.

Gene name	Location	Description	Studies
Genes present on chromosome 21			
DSCAM	21q22.2	• Involved in nervous system development	Abnormal endocardial cushions causing AVSD in cases of overexpression of genes present in chromosome 21
Collagen VI	21q22.3	• Encoded by COL6 A1 and COL6 A2 • Expressed in foetal heart during gestation period • Involves in the development of AV septum	Overexpression of COL6A1and COL6A2 is associated in the formation of AVSD in DS
RCAN1/DSCR1	21q22.1	• Encodes calcineurin inhibitors • Calcineurin dephosphorylates the NFAT y expressed in endothelial tissue of heart	The overexpression of DSCR1in DS cases reduces the dephosphorylation of NFAT causing CHD
KCNJ6	21q22.13	• Encodes G protein-regulated potassium channels	Overexpression can change cardiac regulation
Genes present on other chromosomes			
CRELD1	3p25	• Expressed during endocardial cushion development • CRELD1 is involved in the formation of AVSD in DS cases	Missense mutations in CRELD1 in DS with AVSD cases whereas isolated AVSD cases showed no such mutations
CRELD2	22q13	• CRELD2 involved in embryonic development	–
BMP	1q	• Associated signalling pathways in BMP are involved in number of biological process in cardiovascular development	–
ALK2	2q24.1	• Encodes the type I receptor for BMPs • Involved in endocardial cushion development	• ALK2-knockout decreases the phosphorylation of BMP that disrupts the endocardium cushion formation • ALK mutations in a DS cases having ASD displayed reduced BMP signalling pathway

modified to DSCR that interacts with highly restricted DSCR (HRDSCR) (Korenberg *et al.* 1992). HRDSCR has found its importance in the identification of pathogenic genes. Therefore, knowledge of the underlying molecular mechanism of DS-associated CHD along with the causative genes, variants, and signal pathways is required to be further explorated to provide better diagnostic and management output of these clinical conditions (Pelleri *et al.* 2017; Ackerman *et al.* 2012).

The potential molecular mechanism associated with the occurrence of CHD in DS cases of following reasons are as follow:

CHD caused due to causative genes, SNP and specific miRNA

Candidate genes known to cause CHD in DS cases are COL6A1 (Davies *et al.* 1993), COL6A2 (Fuentes *et al.* 1997); RCAN1 (De La Pompa *et al.* 1998), DSCAM (De La Pompa *et al.* 1998; Barlow *et al.* 2001), KCNJ6 (Lignon *et al.* 2008), RELD1 (Maslen *et al.* 2006a), CRELD2 (Maslen *et al.* 2006b), and ALK2 (Joziasse *et al.* 2011). Genes including *COL6A1* and *A2*, *RCAN1*, *DSCAM*, and *KCNJ6* located on chromosome 21 are involved in embryonic cardiogenesis. The pathogenic mutations in these genes can further cause abnormal heart development. Also, due to the gene dosage mechanism acting on these patients can in turn further magnifies the effects of these pathogenic mutations in the above-mentioned genes in patients. *CRELD1*, *CRELD2*, and *ALK2* genes are on the other hand present on another chromosome that also reported to cause CHD in DS cases due to the introduction of locus-specific mutations. A recently published study used NGS-platform to identify a candidate gene causing CHD in patients with DS where they recruited 240 DNA samples from patients comprising 100 cases of CHD, 110 cases of DS have patients with CHD and 30 cases of isolated DS. They confirmed that pathogenic variants in genes namely: GUSB, GATA3, FLNA, KCNH2 and ENG are the risk factors for the occurrence of CHD in DS

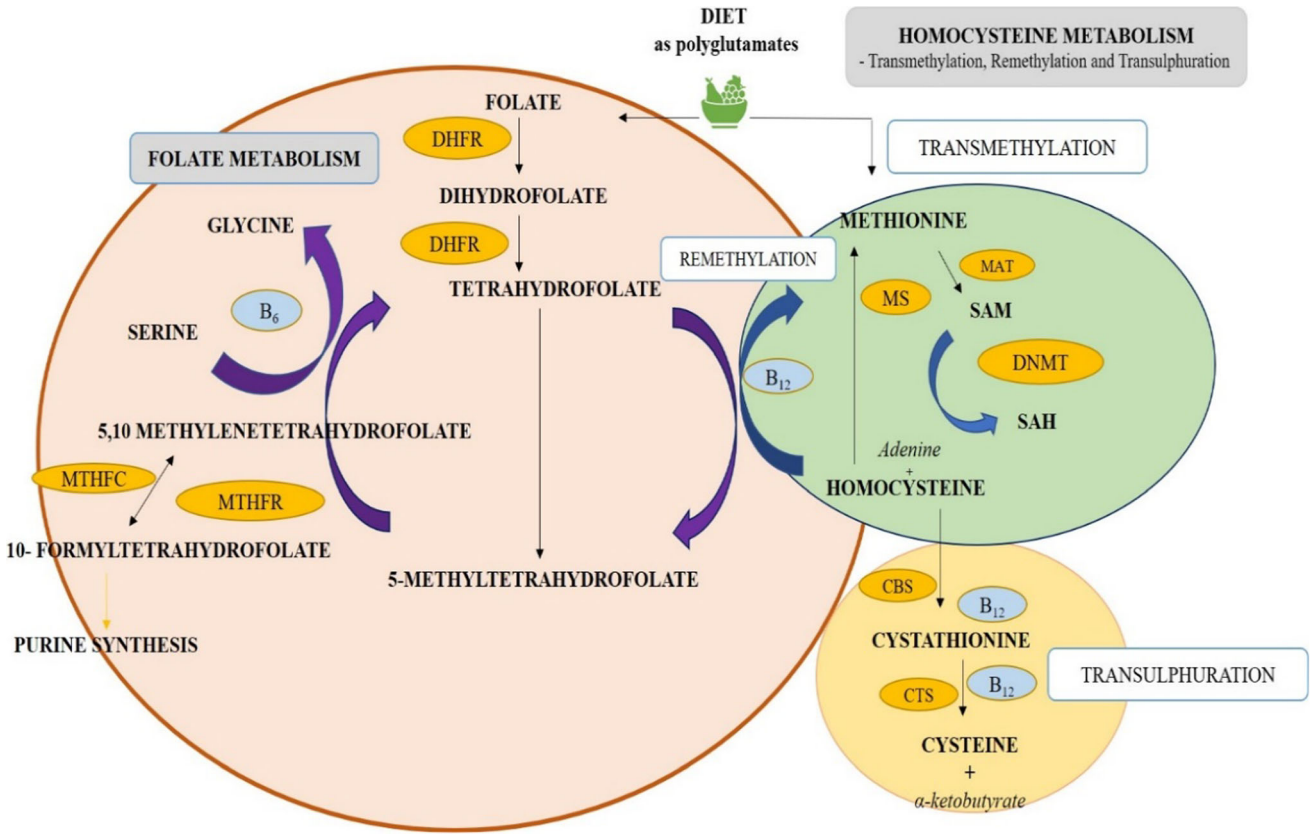

Figure 1. Folate/homocysteine metabolic pathway. The Diagram explains the enzymes, metabolites and cofactors. Metabolites: Homocysteine (Hcy), Methylenetetrahydrofolate (MTHF), Dihydrofolate (DHF), Tetrahydrofolate (THF), S- adenosyl homocysteine (SAH), S-adenosyl methionine (SAM). Enzymes: Methylenetetrahydrofolate Reductase (MTHFR), Methylene Synthase (MS), Cystathionine β Synthase (CBS), Methionine Adenosyltransferase (MAT), Dihydrofolate Reductase (DHFR). Cofactors: vitamin B2 and B6. Liver maintains the level of Hcy by degrading methionine. Hcy is remethylated through the MTR enzyme. The liver employs MAT, CBS, CTH enzymes for this purpose. High levels of methionine in the liver results in increased concentration of SAM, also a potent inhibit of MTHFR enzyme, which in turn activates CBS activity. Higher levels of methionine cause the degradation of Hcy via the transulphuration pathway. Conversely, lower levels of methionine, results in reduced concentration of SAM, which will no longer activate CBS or inhibit MTHFR, further leading to conversation of homocysteine to methionine through remethylation cycle. (a) Transmethylation: MAT catalyzes the synthesis of SAM from methionine and produces SAH, which is also an inhibitor for methyltransferase enzymes. S-adenosylhomocysteine hydrolase (SAHH) further hydrolyses SAH to Hcy and adenosine. (b) Remethylation: remethylation step in Hcy metabolism serves as a linkage between Hcy metabolism and folate cycle. MTR enzyme performs the remethylation of Hcy to methionine using vitamin $B_{12,}$ as a cofactor resulting in the formation of complex, CbI(I)MTR. CbI(I)MTR binds to 5-methTHF. MTRR remethylates and reactivates CbI (II)MTR, utilizing SAM, as a methyl group donor. (c) Transulphuration: transulphuration reaction is carried out by two vitamin-B_6 dependent enzymes: CBS and CTH. CTH is formed by the accumulation of Hcy and serine by CBS enzyme and further, cystathionine is hydrolysed to form cysteine and α-ketobutyrate by enzyme CTH. CBS is involved in protein synthesis where cysteine is a precursor of glutathione, a strong antioxidant.

cases (Alharbi *et al.* 2018). Table 2 shows the details of genes involved in the occurrence of CHD in populations with DS.

Gene dosage effect can also produce various copy number variation and SNP's on chromosome 21 (Sailani *et al.* 2013). Studies have indicated the unequal distribution of various sub-types of CHD present in DS affected individual in different ethnic groups, for examples AVSD is the most reported among Caucasian population (Park *et al.* 1997) while VSD, is commonly seen among Asians (Lo *et al.* 1989). This difference could be because of specific SNP in that particular ethnic group creates a variation in the CHD sub-types in various ethnicities.

MicroRNA (miRNA), ∼20 nucleotide in length, primarily interacts with 3'UTR of mRNA, interrupting the translation processes. The chromosome 21 specific miRNA are namely miR-802, miR-155, miR-99a, miR-125b-2 and let-7c, found to be overexpressed in the tissues present in the heart of DS patients. An overexpression of miRNA-99a/let-7c clusters in DS foetal heart tissue have been reported which further gives the option of utilising specific miRNA as biomarkers in the future for the diagnosis of DS-associated CHD (Wang *et al.* 2016).

Folic acid supplementation

Water-soluble vitamin known as folate or vitamin B_6 is obtained from dietary supplements, green leafy vegetables,

and other fortified food (Blom 2009). Folic acid is a stable form of folate, required mainly for nucleic acid synthesis (Pitkin 2007). Inadequate folate uptake leads to biochemical changes in one-carbon metabolism resulting in hyperhomocysteinaemia or DNA hypomethylation, which in turn results in developing an increased risk of various chronic diseases and developmental disorders (Marti-Carvajal *et al.* 2009). The use of folic acid during pregnancy was found to be associated with AVSD in DS offspring (Kosaki *et al.* 2005). The fate of folate inside the human body is divided into two steps: (i) folate consumption and transportation, (ii) folate/homocysteine (Hcy) metabolism.

***Folate consumption and transportation*:** Folates naturally exist as polyglutamates that are converted to monoglutamates and is then carried by the intestinal apical brush-anchored enzymes, folylpoly-γ-glutamate carboxypeptidase (FGCP). Proton-coupled folate receptor (PCFT1) absorbs monoglutamylated folates in the stomach. 5-Methyl THF is the circulatory form of folate in the plasma and is carried out to the cells by a glycosylphosphatidylinositol-linked glycoprotein receptor, known as folate receptor alpha (FR-α) (Kosaki *et al.* 2005; Pitkin 2007; Blom 2009; Marti-Carvajal *et al.* 2009). Reduced folate carrier (RCF, also called as SLC19A1) is involved in the transport of 5-methyl THF. The SNP rs1051266 (c.80A > G), in RFC gene is reported to obstructs the folate uptake mechanism and poses a risk of the occurrence of CHD in patients with DS (Locke *et al.* 2010). The crucial enzymes involved in the folate/Hcy pathway include methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR), cystathionine b-synthase (CBS) and methionine synthase (MTR).

***Folate/Hcy metabolism: an overview*:** Liver is the chief organ that maintains homocysteine (Hcy) level by degrading the excess amount of methionine. Hcy, is remethylated through MTR enzyme. Liver utilizes specialized group enzymes, for this process including methionine adenosyltransferase (MAT), CBS, cystathionine g- synthase (CTH), glycine N-methyltransferase (GNMT) and betaine homocysteine methyltransferase (BHMT) (Kotb *et al.* 1997; Kosaki *et al.* 2005; Marti-Carvajal *et al.* 2009; Locke *et al.* 2010). High levels of methionine in the liver results in the increased concentration of S-adenosylmethionine (SAM). SAM on the other hand, is also an inhibitor of MTHFR enzyme, which in turn activates CBS activity (Mato *et al.* 1997). Higher levels of methionine cause the degradation of Hcy via the transulphuration pathway. Conversely, lower levels of methionine, results in reduced concentration of SAM leading to conservation of Hcy via remethylation back to methionine. Figure 1 shows the detailed description of folate/Hcy metabolism divided into three steps: namely transmethylation, remethylation, and transulphuration. Brandalize *et al.* (2009) recruited 57 mothers of DS babies having CHD that carried MTHFR677 CT/TT genotypes. These mothers did not take folic acid during the pregnancy. The study showed to have a 2.26-times increased risk of giving a DS having CHD thus further supporting the hypothesis that genetic variants present in MTHFR genes leads to the occurrence of CHD in DS offsprings. Presence of maternal T allele MTHFR C677T polymorphisms can also pose a risk for the occurrence of CHD in DS offspring. Hence it is predicted that those mothers having this variant could be helped from the use of folic acid during pregnancy to reduce the risk of occurrence of CHD in DS affected babies (Brandalize *et al.* 2009). A study reported that the risk of having AVSD (a subtype of CHD) in DS babies was higher in the absence of maternal folic acid intake during pregnancy (OR = 1.69, 95% CI = 1.08−2.63) when compared to DS without CHD (Brandalize *et al.* 2009). Thus, maternal folic acid intake during pregnancy is probably associated with a reduced risk of CHD in DS offspring. The study also showed a substantial difference in the methylation levels of DNA isolated from two sources, i.e., from blood and from the heart tissue of DS affected foetuses. Additional, 22 DNA samples were isolated from foetal heart tissue of DS presented increased methylation levels when compared to healthy foetuses (Serra-Juhé *et al.* 2015).

Increased methylation levels in MTHFR promoter regions were seen in DNA isolated from patients of various diseases including cancer, cardiovascular or renal disorders, and also seen in women having pre-eclampsia. These increased methylation levels can cause decreased MTHFR protein activity, further leading to the risk of developing a cardiovascular disease (Khazamipour *et al.* 2009; Wei *et al.* 2015; Vaissière *et al.* 2009; Asim *et al.* 2017a, b; Coppedè 2015; Antonarakis 2017). In our previous study, we have explored the methylation levels of CpG islands in the promoter region of MTHFR gene in three groups including DS mothers having CHD affected offspring, DS mothers without CHD offsprings and mothers having history of delivering healthy offspring (Asim *et al.* 2017a, b). A few studies have also been conducted on another important enzyme involved in folate/Hcy metabolism, methionine MTRR enzyme, encoded by MTRR gene, showed that the genetic polymorphism in this gene is also associated with the occurrence of CHD in DS offspring (Zeng *et al.* 2011; Brandalize *et al.* 2009; Bean Lora *et al.* 2011; Asim *et al.* 2017a, b). The hypothesis of MTHFR promoter methylation serves as one of the crucial risk factors in mothers of DS leading to the birth of CHD offspring still required to be confirmed on large-scale sample sizes in different ethnic groups.

Based on the above-conducted survey, the pathogenic variants in the maternal subjects located on candidate genes, namely SLC19A1, MTHFR and MTRR are involved in folate/Hcy metabolism, found to be associated with the occurrence of CHD in DS cases. Thus, the screening of the polymorphisms present in above-mentioned candidate genes involved in the folate/Hcy metabolism in maternal subject can further be used in the analysis of the occurrence of CHD with DS. Hence, the current study also highlights how the interaction between the candidate genes with genetic

polymorphisms and folate/Hcy metabolism can lead to the pathogenesis of the disease.

Signalling pathways

Numerous pathways including VEGF-A (Ackerman *et al.* 2012); Sonic Hedgehog (Shh) signalling (Bean Lora *et al.* 2011), the methionine salvage pathway (Ripoll *et al.* 2012), the folate/Hcy pathway (Fuentes *et al.* 2000; Locke *et al.* 2010; Ripoll *et al.* 2012; Chen *et al.* 2016) and the calcineurin/NFAT (Locke *et al.* 2010) pathways have also contributed to the existence of CHD in DS cases. A study revealed that genes identified as risk factors for AVSD in DS cases, namely CRELD1, COL6A1, COL6A2, FBLN2, FRZB and GATA5 (Ackerman *et al.* 2012) are involved in the VEGF-A pathway. AVSD is caused possibly due to disturbances in theses central genes involved in VEGF-A pathway. Signalling pathways including Hedgehog and Sonic Hedgehog (Shh), are reported to play an important role in the formation of AVSD in DS cases. Hedgehog signalling is involved in embryonic development, while abnormal expression in Shh signalling inhibits the fusion of AV cushion resulting in AVSD cases (Chen *et al.* 2016; Briggs *et al.* 2012). Alternatively, *CRELD1* gene is reported to act as a regulator of calcineurin/NFATc1 signalling, which is crucial for regulating cardiac development by dephosphorylation of the transcription factor nuclear factor of activated T cells (NFAT) (Chen *et al.* 2016).

Diagnosis of CHD

Prenatal diagnosis

Prenatal diagnosis focus on detecting the problem in the foetus during pregnancy to provide better prenatal care and can be of following two types: (i) preimplantation genetic diagnosis (PGD) and (ii) prenatal screening. Preimplantation genetic diagnosis aims to screen the genetic abnormalities present during the implantation phase of foetal development. PGD is beneficial in assessing the risk of passing down any genetic abnormalities from parents to offspring. PGD has been used in many genetic diseases such as inherited cardiac conditions such as Marfan syndrome, dilated cardiomyopathy, HCM, and muscular dystrophies (Vermeesch *et al.* 2016; Traeger-Synodinos 2017). The study was approved by the institutional ethics committee (Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, India IEC code: 2018-100-EMP-104).

Cell-free DNA screening

Noninvasive prenatal diagnosis (NIPD), also known as foetal cell-free DNA (fcfDNA) screening was available for clinical settings in 2011 (Gregg *et al.* 2016). The maternal serum is isolated from the whole blood of pregnant women through centrifugation followed by separation of fcfDNA. Once the fcfDNA is separated, the ratio of DNA can be evaluated between aneuploidies, and other chromosomes using both bioinformatic analysis and NGS approach. NIPD has a 5% chance of giving a false-positive result and the detection rates are ∼50–90%. The ACMG guideline recommends that all pregnant women should be informed the sensitivity of NIPD (fcfDNA screening) for chromosomal abnormalities like trisomy 21, 18, and 13, and offering the test to high-risk pregnancies with advanced maternal age or presence of any foetal abnormalities indicated during ultrasound finding (Committed on Genetics 2016). Also, in the presence of any CNV, the guidelines also recommend the fcfDNA analysis for detecting sex chromosomal abnormalities and CNVs and further the test should be confirmed through chorionic villus sampling or amniocentesis. In case of any abnormalities present in the echocardiograms, and ultrasound findings, then, the American College of Obstetricians and Gynaecologists and the Society of Maternal-Fetal Medicine recommend the use of prenatal chromosomal microarray along with the invasive prenatal diagnosis (Donofrio *et al.* 2014).

Foetal echocardiography

Detection and characterization of congenital heart malformations can be done by widely used technology, foetal echocardiography (FE). FE aims to evaluate the risk factors identified by ultrasound screening and is conducted in maternal cases with poor obstetric history including advanced maternal age, presence of diabetes mellitus/autoimmune condition, use of certain drugs during pregnancies, exposure of toxins, abnormalities in the umbilical-placental development (e.g., single umbilical artery and monochorionic twinning) and specific cases known to carry certain heritable genetic conditions (Copel *et al.* 1986; Malik *et al.* 2007; Bilardo *et al.* 2001; Merker *et al.* 2018). FE is also used in the case of pregnancies with a history of any congenital cardiac defect in first—a degree family relative. FE is also used to diagnose the abnormalities associated with central nervous system such as microcephaly, hydrocephaly, agenesis of the corpus callosum); digestive system abnormalities such as oesophageal or duodenal atresia, diaphragmatic hernia, or omphalocele/gastroschisis; structural abnormalities in kidneys, craniofacial structures, or limbs. FE can also analyse the risk factors for CHD including any unexplained growth delay and presences of features such as the increased thickness of nuchal translucency in ultrasound (Copel *et al.* 1986; Bilardo *et al.* 2001; Malik *et al.* 2007; Merker *et al.* 2018).

Array CGH (aCGH)

An aCGH evaluates CNV between DNA from two sources, one being the patients' DNA (test sample) while the other is

the control DNA to confer microdeletions and microdeletions existing among them. An aCGH does not require culturing of cells and can evaluate CNV with large insertions or deletions of DNA upto100 kb nucleotides in length when used in conjugation with DNA microarray (Chiu *et al.* 2008).

NGS-based approaches

Nowadays, with the introduction of deep sequencing has revolutionized the era of genomic research, allowing us to sequence the entire human genome (WGS) in a short time including all exons (WES) or selected sets of genes (target gene). All the platforms of NGS undergo massive parallel sequencing and bioinformatic analysis is used to further map individual reads according to the human genome reference. The gene panel tests either use hybridization or PCR approach to capture the regions of genes (typically 1–100 genes) that encode for protein sequences. In WES, it captures all 18,000 exons present in human genes ($\approx$1.5% of the entire genome) by using hybridization method, while in WGS, it obtains genetic information from the entire human genome without a complex capture process followed by NGS. The NGS can detect SNPs and INDELS in cases with unexplained pathophysiology causing CHD (Chiu *et al.* 2008). Currently, in the NGS setting, the sample size is $\approx$100–250 nucleotides and in case of long-read sequencing it is more than 10,000 nucleotides. In the near future, this technique will be mostly used for long-read sequencing that can detect SNPs/INDELs and CNVs simultaneously using a single test (Chiu *et al.* 2008; Xu *et al.* 2018).

Nowadays, NGS-based approach can be used in the prenatal setting to sequence the foetal cell-free DNA (fcfDNA) as a screening tool for aneuploidies and biomarkers for CHD as well (Xu *et al.* 2018). However, this technique offers low sensitivity and specificity for aneuploidy relative when compared with the gold standard tests such as FISH testing of amniotic fluid, but in the near future it can be used to detect novel candidate genes for CHD during foetal life (Chiu *et al.* 2008). A study was reported in 2018 that highlights the rationality, practicability and analysis strategy of NGS for the detection of variants in families having nonsyndromic AVSDs in DS foetus by using WGS to detect CNVs and WES to detect the gene mutations in cases without AVSD-associated CNVs. A total of 1736 CNVs were detected from 50 foetuses with AVSD, of which 17 *de novo* CNVs were selected having 10 causative candidate genes for AVSD from AVSD foetuses and none of the CNVs were reported from healthy foetus. These 10 candidate genes were retrieved in DECIPHER, of which seven genes, namely *NOTCH2*, *NIPBL*, *EHMT1*, *NR2F2*, *TBX1*, *SHANK3*, *SMC1A* were detected in patients with CHD subtypes such as AVSD, ASD and VSD (Resta *et al.* 2006).

Genetic counselling

Genetic counselling is a practice of guiding the patients and their relatives; and also associated families, of any underlying genetic disorders or its risk factors for the better understanding of the disease and its progression. It aims (i) to provide the risk assessment of occurrence and recurrence of any genetic disorder in cases having previous medical history, (ii) to provide awareness about the inheritance pattern of the disease putting emphasis on testing for the disease, and (iii) to provide informed consent choices from the patient, families, or guardians. Nowadays, a genetic counsellor in cardiovascular genetics has become a helpful asset in providing the recurrence risk in obtaining the medical history of family, in prenatal diagnosis, facilitating the appropriate diagnostic facility, interpretations of results and providing referrals to super specialist clinical geneticists. These super-specialists are the team of experts conducting medical services in the field of prenatal diagnosis, dysmorphology, metabolic disorders, Mendelian disorders, bioinformatic results interpretation, genomics, and proteomics. Previous studies have shown that genetic counselling done by medical geneticist not only increases the diagnostic rate of various syndromes associated with CHD but also these patients are seen in the regular follow-up for cardiac evaluation (Resta *et al.* 2006; Ahrens-Nicklas *et al.* 2016; Goldenberg *et al.* 2017). Genetic counselling can be very useful not only during prebirth but also have significant benefits in the management of CHD in DS cases afterbirth. In the case of DS babies already born with CHD, the genetic counselling can help in monitoring the prognosis of diseases by conducting regular check-ups and echocardiography. In severe cases, surgery can be advised for such cases thus helping in the better management of the disease.

Conclusion

CHD are a multifactorial disorder that accounts for 50% of all DS cases (Weijerman *et al.* 2008). AVSD in DS is reported to be highly heterogenic in nature and the highest prevalence among all sub-types of DS associated-CHD. Approximately 64% of AVSD cases are associated with genetics syndrome while 36% account for nonsyndromic cases (Digilio *et al.* 1998). The syndromic AVSD is further sub-dived into syndromic AVSD cases with chromosomal anomalies and syndromic AVSD with monogenic disorder (for details see table 1).

The present review focusses on the possible molecular mechanism in DS cases leading to the occurrence of CHD along with the advancement of the emerging diagnostic techniques for such patients. We have described in detail about the two possible mechanisms, namely gene dosage effect and gene mutation hypothesis, causing CHD in patients with DS babies. We have also shed light on the causative genes for DS associated CHD located either on

chromosome 21 or outside chromosome 21. Along with locus-specific mutation, numerous SNP and CNV are also reported to contribute in the formation of CHD in patients with DS (Sailani *et al.* 2013). The role of several miRNAs is also described to cause CHD associated DS (Wang *et al.* 2016). The association of use of maternal folic acid during pregnancy in DS was first introduced by James and Hobbs *et al.* in the late 90s. Nowadays, a few studies have been conducted in this aspect that showed lack of folic acid usage during pregnancy is one of the maternal risk factors associated with the development of CHD in DS cases. We have also discussed about the signalling pathway that contributed for the development of CHD in DS cases (Malone *et al.* 2005).

With the help of both, our understanding of the mechanism of the pathogenesis of CHD in patients with DS along with the availability of emerging new technologies for their diagnosis has facilitated a novel gene discovery that has ultimately provided us a better prognosis, treatment and management for such diseases. Accurate diagnosis and treatment are now available with the introduction of CNV detection and NGS based approaches such as WES, WGS, target sequencing and sequencing of fcfDNA by the medical geneticist and cardiologist have allowed us for further identification of familial recurrence risk and relatives who are at risk of this disease after genetic counselling, thereby providing reproductive options and improving proper care of DS associated CHD. Nowadays, WES is widely used by medical geneticist and cardiologist in patients having CHD-related syndromes when no pathogenic diagnosis is available after performing several other diagnostics tests (Resta *et al.* 2006; Chiu *et al.* 2008; Xu *et al.* 2018).

Future research

Based on the above observations, the need of WGS can be performed on a larger sample size with the aim of identifying new genetic risk factors and candidate genes for our better understanding of conditions associated with CHD-DS. Functional studies on animal models are also required to explore other possible pathogenic mechanisms causing CHD in the population with DS. Gene editing tools in cardiac tissues can be used to further identify the new possible pathological mechanisms and signalling pathways associated with DS-CHD. Induced pluripotent stem cell approaches can also be used in the future to discover new drugs through cell therapy-based strategies.

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