Review

Genetic factors in non-syndromic congenital heart malformations

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The genetic defect in most patients with non-syndromic congenital heart malformations (CHM) is unknown, although more than 40 different genes have already been implicated. Only a minority of CHM seems to be due to monogenetic mutations, and the majority occurs sporadically. The multifactorial inheritance hypothesis of common diseases suggesting that the cumulative effect of multiple genetic and environmental risk factors leads to disease, might also apply for CHM.
We review here the monogenic disease genes with high-penetrance mutations, susceptibility genes with reduced-penetrance mutations, and somatic mutations implicated in non-syndromic CHM.

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Congenital heart malformations (CHM) are among the most common human congenital defects, occurring in 6 to 8 out of 1000 live-births (1). The majority of CHM with monogenic inheritance is associated with non-cardiac malformations, and thereby constitutes syndromic forms of CHM. These include well-known examples such as Holt-Oram syndrome, Alagille syndrome, and Noonan syndrome, among many others (for review, see Refs 2–4). Many of these syndromes have a monogenic mode of inheritance. In contrast, most non-syndromic CHM occurs sporadically, and families with clear monogenic inheritance of non-syndromic CHM are scarce (5–8). This makes the identification of human disease genes involved in non-syndromic CHM by a classical positional genetics approach difficult. The sporadic nature of most non-syndromic CHM is traditionally explained by the multifactorial inheritance model which involves a multitude of susceptibility genes with low-penetrance mutations (common variants)

or intermediate-penetrance mutations (rare variants) superposed on unfavorable environmental factors (9). Although widely accepted, this hypothesis remains difficult to prove, and only a handful of studies on accumulating and/or interacting effects in CHM have been reported (10–13).

Here we review the different genetic factors implicated in the development of non-syndromic CHM, including disease genes with high-penetrance mutations, susceptibility genes with intermediate- or low-penetrance mutations, and somatic mutations.

Disease genes with high-penetrance mutations

Many syndromic forms of CHM exist, and for many the primary gene defect has been identified. In recent years an increasing number of families with monogenic forms of non-syndromic CHM have been reported, which has facilitated the positional cloning of several disease genes, including *ZIC3*, *GATA4*, *NKX2.5*,

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NKX2.6, *MYH6*, *ACTC1*, and *NOTCH1*. Other disease genes were found through a candidate gene approach: these include *TBX1, TBX20, CFC1, CITED2, CRELD1, FOG2, LEFTY2, NODAL, GDF1, FOXH1, TDGF, MYOCD, TLL1, THRAP2* and *ANKRD1* (Table 1). The majority of monogenic forms of non-syndromic CHM are caused by a single high-penetrance autosomal dominant mutation. Nevertheless, the majority of mutations reported in many of the human CHM genes are missense mutations of which the pathogenic, let alone the monogenic nature, has not been formally showed, and some of these mutations have reduced (intermediate or low) penetrance (Table 2).

Many of the genes implicated in non-syndromic CHM are transcriptional regulators of heart morphogenesis. The fetal developmental program of the heart involves multiple pathways with extensive cross-talking and promiscuous ligand–receptor interactions, secondary signal transduction pathways and a network of transcription factors that determines the expression of cardio-specific effector genes (Fig. 1). Various ligands in the circulation or the extracellular space of the heart, including hormones, cytokines, and growth factors, stimulate receptors in the cell membrane of cardiac cells. These ligand–receptor complexes include JAGGED/NOTCH, TGFB-BMP/TGFBR,

Table 1. Disease-causing mutations with presumed high penetrance contributing to non-syndromic CHM

Genes	Cardiac phenotypes	Mutations[a]	References
Ligands–receptors			
NOTCH1	BAV, AS	R1108X, H1505del, T596M, P1797H, P1390T, A683T, G661S	(19–22)
CFC1	Heterotaxy	R112C, R189C	(29, 30)
	TGA	Splice donor site duplication intron 4	(38)
	TOF	IVS4+2T>C	(10)
	TA	IVS4+2T>C	(10)
	AVSD	IVS4+2T>C	(10)
LEFTY2	Heterotaxy	R314X, S342K	(27)
ACVR2B	Heterotaxy	R40H, V494I	(28)
GDF1	TOF	G162D, S309P, P312T	(26)
ALK2	ASD	L343P	(36)
	TGA	C227X, A318T	(26)
	DORV	C267Y	(26)
	AVSD	G262S, R68H	(26)
NODAL	Heterotaxy	E203K, G260R, R275C, V284F, R234_P241delinsLTS, IVS1-1G>T, IVS2+1G>A	(18)
TDGF1	TOF	P125L	(10)
JAG1	PS, TOF	G274D, E228fs	(110, 111)
Transcription factors			
GATA4	ASD	S52F, G296S, S358del, E359fs, Q316E, A411V	(8, 42–45, 48, 185, 186)
	TOF	E216D, D425N, A118_A119insA, P407Q	(45, 46, 48)
	ASD ± PS	G296S, S358fs, G296C	(8, 43, 44)
	VSD	A411V, E359K, A6V, S46del, A125_A126insAA, S429T, A422V	(45, 46)
	HRV	L403M	(47)
	PAPVR	A411V	(186)
GATA6	PTA, PS	E486del, N466H	(53)
NKX2.5	ASD-AV block	Q149X, R189G, T178M, Y259X, Q170X, Q198X, Q160P, IVS1+1G>T+1T, c.215_221del7, A75fs, A88fs, R190C, Y256X, Q170X, E160P, Y256X, K104fs, A127E, R142C, Q817H, N188K, R189G, Y191C, c.701_702ins5, C264X, E109X	(5, 42, 55–61, 63, 187–190)

Table 1. Continued

Genes	Cardiac phenotypes	Mutations[a]	References
	TOF	Q22P, R216C, R142C, A323T, Q149X, R25C	(55, 61–63, 191–193)
	HLHS, CoA, IAA	T178M, R25C, P275T	(59, 63)
	Heterotaxy	c.215_221del7	(57)
	TGA	A63V	(63)
	DORV	N291del	(63)
	VSD	Y191C, Q149X, Y259X, E109X	(61, 190)
	Ebstein	A42P	(191)
NKX2.6	PTA	F157L	(66)
TBX20	ASD, CoA	I152M, Q195X	(7)
	VSD	I152M	(7)
	PDA	I152M	(7)
	DCM	Q195X	(7)
	MS, HLV	Q195X	(7)
	ASD	I121M	(68)
CITED2	VSD	S170_G178del	(71)
	ASD	G178_S179del, S198_G199del	(71)
FOXH1	TOF	D350G, P336L, S339G	(10)
	CHM	S16L, G267R, T242I, D328E	(10)
ZIC3	Heterotaxy	Various mutations	(114, 194)
	TGA	W255G, K467X, K405E	(112–114)
	ASD, PS	A217P	(114)
TBX5	ASD, VSD, AVSD	G80R	(195)
TBX1	VSD	A379_G381del	(120)
	IAA	A466_A476dup c.1399-1428dup	(121, 193)
ANKRD1	TAPVR	T116M	(73)
Contractile proteins			
MYH11	PDA, aorta aneurysm	L1456_N1526del, R1241_L1264del	(74)
ACTC1	ASD, VSD	M123V, c.215_231del17, E101K, G99L	(15, 86, 87)
MYH6	ASD	I820N	(14)
MYH7	ASD, Ebstein	R281T, F230S	(16)
MYBPC3	ASD, VSD	Various mutations	(81–83)
Miscellaneous			
FLNA	XMVD	G288R, V711D, P637Q, deletion exons 16–19	(99)
ELN	SVAS	Various mutations	(104)
TLL1	ASD	M182L, A238V, L627V	(108)
THRAP2	TGA	R1872H, D2023G	(107)

AS, aortic valve stenosis; ASD, atrial septal defect; AV, atrioventricular; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; CHM, congenital heart malformation; CoA, coarctation of the aorta; DORV, double outlet right ventricle; HLHS, hypoplastic left heart syndrome; HLV, hypoplastic left ventricle; HRV, hypoplastic right ventricle; IAA, interrupted aortic arch; MS, mitral valve stenosis; NS, not specified; PA, pulmonary atresia; PAPVR, partial anomalous pulmonary venous retour; PDA, patent ductus arteriosus; PS, pulmonary valve stenosis; PTA, persistent truncus arteriosus; RV, right ventricle; SVAS, supravalvular aortic stenosis; TAPVR, total anomalous pulmonary venous retour; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect; XMVD, X-linked myxomatous valvular dystrophy.
[a]Mutations in the open reading frame are described at the protein level.

VEGF/FLT1-FLK1, NODAL/ACVRA-ACVRB, and RTK/RAS. These membrane complexes (in)-activate different signal transduction pathways converging on a network of transcriptional factors and regulators. Phosphorylation/dephosphorylation by kinases such as mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase 1/2 (ERK1/2), cJUN, GSK, and calcineurin further controls these transcriptional networks. The transcriptional regulators of heart morphogenesis include several T-BOX transcription factors (TBX1, TBX5 and TBX20), various GATA transcription factors (GATA4, FOG2), myocyte enhancer factor 2 (MEF2), nuclear factor of activated T cells (NFAT), serum response factor (SRF), homeobox transcription factors (NKX2.5, NKX2.6),

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Table 2. Rare variants with intermediate penetrance contributing to non-syndromic CHM

Gene	Function	Cardiac phenotypes	Intermediate-penetrance mutations[a]	References
CRELD1	Matricellular protein	AVSD	R329C, T311I, P162A, E414K	(126–128, 196)
		AVSD, dextrocardia	R107H	(126)
NKX2.5	Transcription factor	TOF	R25C, E21Q, A219V	(62, 63, 192)
		ASD-AV block	K15I	(63)
		DORV	N291del	(63)
		VSD	V50I	(115)
NOTCH1	Transcription factor	LVOTO	A683T, G661S	(22)
GATA4	Transcription factor	ASD	D425N, G93A	(45)
		AVSD	P163S, A346V	(47)
ZIC3	Transcription factor	TGA	G17C	(115)
FOG2	Transcription factor	TOF	E30G, S657G	(130)
FOXH1	Transcription factor	TGA	P21S	(115)
		TOF	V112M, S113T/S346G, S339G	(10)
NODAL	Ligand	Heterotaxy-CHM	G260R	(18)
		TGA	IVS2-1G>C	(115)
THRAP2	Receptor-associated protein	TGA	E251G	(107)
MYOCD	Regulator	PS	K259R	(197)
CFC1	Receptor	TGA	R78W	(38, 134)
		TOF	R78W, G174fs	(10)
		IAA	R78W	(10)
		DORV	G174fs, N21H, R47Q	(38, 134)

ASD, atrial septal defect; AV, atrioventricular; AVSD, atrioventricular septal defect; CHM, congenital heart malformation; DORV, double outlet right ventricle; IAA, interrupted aortic arch; LVOTO, left ventricular outflow tract obstruction; PS, pulmonary valve stenosis; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect.
[a]Mutations in the open reading frame are described at the protein level.

basic helix-loop-helix (bHLH) transcription factors (HAND1, HAND2), and various SMAD transcription factors. These transcription factors regulate the expression of numerous cardiac effector genes, including atrial natriuretic factor (ANF), b-type natriuretic peptide (BNP), myosins including α-myosin heavy chain (α-MHC) encoded by the MYH6 gene, and cardiac actin encoded by the ACTC1 gene.

Most high-penetrance mutations occur in two different groups of genes, e.g. transcription factors-regulators and cardiac effector genes. Several transcriptional regulators, including GATA4, FOG2, NKX2.5, NKX2.6, ZIC3, CITED2, TBX1, and TBX20, have been implicated in non-syndromic CHM. Recently, also mutations in sarcomeric protein genes known to be involved in cardiomyopathies MYH6 (14), ACTC1 (15) and MYH7 (16) have been shown to cause various CHM (17). Conversely, mutations in transcription factors such as NKX2.5 and TBX20 have been shown to cause cardiomyopathies.

A similar signal transduction pathway, referred to as the NODAL signal transduction pathway, is involved in the establishment of left–right (LR) asymmetry: NODAL, LEFTY1/LEFTY2 and

GDF1 are ligands for a receptor complex consisting of CFC1, TDGF1, ACVR2A/ACVR2B and ACVR1B. This complex determines the activity of transcription factors including FOXH1 that have cardiac-specific downstream targets such as PITX2 (Fig. 1). Mutations in the NODAL pathway are not only involved in laterality defects but also in heterotaxy-related CHM such as tetralogy of Fallot (TOF), transposition of the great arteries (TGA) and double outlet right ventricle (DORV) (18).

The different disease genes with high-penetrance mutations implicated in non-syndromic CHM are discussed below (Tables 1 and 3).

Ligands and receptors

NOTCH1
Bicuspid aortic valve (BAV) ± severe valve calcification, the most common CHM, can be caused by autosomal dominant mutations in the *NOTCH1* gene in a minority of patients (19). BAV may be part of left ventricular outflow tract obstruction (LVOTO) that can also be caused by *NOTCH1* mutations (20–22) (Tables 1 and 3). NOTCH proteins are single-pass transmembrane receptors that regulate many developmental pathways (Fig. 1). Mutations in the genes encoding NOTCH2 and
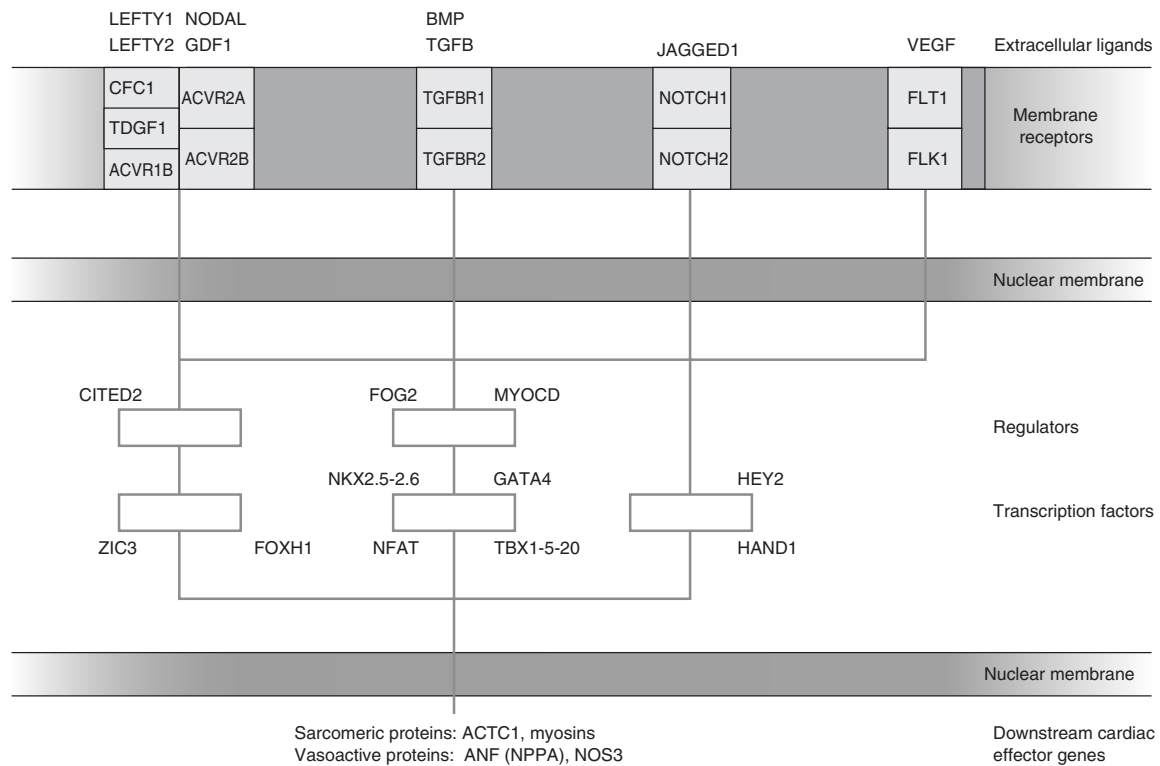
Fig. 1. Signaling pathways in heart morphogenesis involved in non-syndromic CHM. The fetal developmental program of the heart involves many pathways with ligand–receptor interactions, signal transduction pathways and interacting transcription factors that determine the expression of cardio-specific effector genes. The figure is simplified to focus on genes implicated in non-syndromic CHM. The disease genes encode members of all compartments of the pathway, including ligands (*LEFTY2, NODAL, VEGF, GDF1, JAGGED1*), receptors (*CFC1, TDGF1, ACVR2B, NOTCH1*), transcription factors-regulators (*CITED2, TFAP2B, ZIC3, FOXH1, FOG2, MYOCD, NKX2.5-2.6, TBX1-5-20, GATA4, HEY2*), and downstream effectortargets including sarcomeric proteins (*ACTC1, Myosins*) and vasoactive proteins (*ANF, NOS3*). Significant ligand–receptor promiscuity and cross-talking between the different signal transduction pathways exists.

its ligand JAGGED1 lead to Alagille syndrome, a syndromic CHM characterized by peripheral pulmonary artery stenosis and septal defects. Mouse embryos that are double mutant for the Notch1 and Notch2 receptors exhibit defects in LR asymmetry, indicating that the Notch signaling pathway plays a primary role in the establishment of LR asymmetry, thereby directly regulating expression of the *Nodal* gene (23). Up to now no *NOTCH1* mutations have been reported in patients with laterality defects.

NODAL

Five percentage of patients affected with either heterotaxy or heterotaxy-related HCM such as looping defects including TGA and DORV have a mutation in the *NODAL* gene (18) (Tables 1 and 3). In mice Nodal is asymmetrically expressed in the left lateral plate mesoderm, and Nodal signaling specifies left-sidedness by activation of Pitx2. Nodal-deficient mice die prior to the establishment of the LR axis, lack the primitive streak and do

not form mesoderm (24, 25). NODAL, a member of transforming growth factor-beta (TGFβ) superfamily of developmental regulators, is part of the NODAL signal transduction pathway, which regulates the establishment of the LR axis. Mutations have also been found in other components of the NODAL signal transduction pathway, including the *GDF1* (26), *LEFTY2* (27), *ACVR2B* (28), *CFC1* (29, 30), *FOXH1* (10) and *TDGF1* (10) genes (Fig. 1).

GDF1

Mutations in the *GDF1* gene have been found in 2% of a large group of patients with a wide spectrum of CHM, including TGA, DORV, TOF and interrupted aortic arch (IAA) (26) (Tables 1 and 3). Mice lacking Gdf1 exhibit a spectrum of defects related to LR axis formation, including visceral situs inversus, right pulmonary isomerism and looping defects such as TGA and DORV (31). GDF1 is a growth differentiation factor that belongs to the TGFβ superfamily. It is a ligand

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Table 3. Genes involved in non-syndromic CHM

CHM	BAV/AS	VSD	ASD	PS	TOF	AVSD	TGA	HLHS	DORV	Heterotaxy
Incidence[a]	14 / 0.8	4	1.0	0.7	0.4	0.3	0.2	0.2	0.16	0.1
Genes	NOTCH1	NKX.2.5[b]	NKX2.5[b]	GATA4	NKX2.5	GATA4	NKX2.5	NKX2.5	NKX2.5	ZIC3
		GATA4	GATA4	MYOCD	GATA4	CRELD1	THRAP2	NOTCH1	GDF1	GDF1
		TBX20	TBX20	JAG1	TBX1	CFC1	ZIC3	HAND1[c]	CFC1	CFC1
		TBX1	MYH6	GATA6	FOG2	GDF1	CFC1	GJA1[c]		LEFTY2
		ACTC1	ACTC1		CFC1	NKX2.5[c]	GDF1			ACVR2B
		MYBPC3	MYH7		NOTCH1	TBX5[c]	THRAP2			NODAL
		TBX5	MYBPC3		GDF1	HEY2[c]	FOXH1			NKX2.5
		CITED2	TBX5[b]		TDGF1	PTPN11				CRELD1
			CITED2		JAG1	ALK2				FOXH1
			TLL1		FOXH1					

AS, aortic valve stenosis; ASD, atrial septal defect; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; CHM, congenital heart malformation; DORV, double outlet right ventricle; HLHS, hypoplastic left heart syndrome; PS, pulmonary valve stenosis; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect.
[a]Per 1000 live-births.
[b]Both germline and somatic mutations.
[c]Somatic mutations.

of ACVR2, and part of the NODAL signal transduction pathway (32) (Fig. 1).

LEFTY2

In two patients with heterotaxy and left isomerism, mutations in *LEFTY2* have been described, but overall *LEFTY2* mutations are uncommon in heterotaxy (27) (Tables 1 and 3). Mice with targeted deletion of the *Lefty2* asymmetric enhancer (which regulates LR expression of Lefty2) show left isomerism (33). *LEFTY2* and the very homologous *LEFTY1* encode TGFβ-like proteins that are ligands in the NODAL signal transduction pathway (Fig. 1).

ACVR2B

In three patients with heterotaxy mutations in the *ACVR2B* gene have been reported (28) (Tables 1 and 3). *Acvr2b*–/– knockout mice show abnormal LR axis development, ASD and ventricular septal defects (VSD), right-sided morphology of the left atrium and left lung, and spleen hypoplasia (34). *Pitx2*–/– knockout mice have cardiac defects similar to *Acvr2b* knockout mice (35), supporting the evidence that Pitx2 is a downstream target of the Acvr2b signal transduction pathway. ACVR2B belongs to the family of activins, TGFβ-related proteins that act as receptors for ligands such as LEFTY1, LEFTY2, GDF1 and NODAL in the NODAL signaling pathway (Fig. 1).

ACVR1 (ALK2)

A single mutation (L343P) in the *ACVR1 (ALK2)* gene has been found in a patient with primum-type

ASD (36). Injection of L343P RNA into zebrafish embryos results in improper atrioventricular canal formation, whereas mice with an endothelial-specific Alk2 mutation display ASD, resulting from a failure of endocardial cells to differentiate into the mesenchyme in the AV canal (37). The *ACVR1* gene encodes for the activin A receptor type 1 that belongs to the family of activin receptors that are active in the NODAL signaling pathway (Fig. 1).

CFC1

A minority of patients with heterotaxy (29), TGA and DORV (38) or TOF (10) show mutations in the *CFC1* gene (Tables 1 and 3). Mutant *Cfc1* mice have heterotaxy (39). The *CFC1* gene encodes Cryptic, which belongs to the EGF (epidermal growth factor)-CFC family of proteins (consisting of Cripto, Frl1, and Cryptic). These proteins are membrane-associated NODAL coreceptors in the NODAL pathway (Fig. 1).

TDGF1

Two patients with TOF (10) have been reported to have a *TDGF1* mutation (Tables 1 and 3). Targeted disruption of the *Tdgf1* gene is lethal in mice and null mutants die in the absence of a node and primative streak (40). TDGF1 (CRIPTO) is an EGF-CFC family member, which acts as a co-receptor in the NODAL signaling pathway (Fig. 1).

FOXH1

Several patients with CHM (mainly TOF, few with heterotaxy) have been reported to have a *FOXH1*

mutation (10) (Tables 1 and 3). Foxh1−/− mutant mouse embryos fail to form the outflow tract and right ventricle (41). FOXH1 is a forkhead DNA-binding transcription factor in the NODAL signaling pathway. It is essential in the development of the second heart field (SHF) and derivatives (the right ventricle and outflow tract), during looping morphogenesis of the heart (Fig. 1).

Transcription factors and regulators

GATA4

Mutations in the *GATA4* gene have been reported in familial cases of ASD ± pulmonary stenosis (PS) (8, 42–44), and in a minority (1–4%) of sporadic patients with septal defects or conotruncal anomalies (45–48) (Tables 1 and 3). Homozygous *Gata4* knockout mice die in utero and develop two symmetric promyocardial primordia that fail to migrate ventrally and form two independent heart tubes (49, 50). Mice with heterozygous *Gata4* mutations exhibit septal defects and endocardial cushion defects (47). The different members of the GATA zinc-finger transcription factor family (GATA1-6) recognize the consensus target sequence (T/A)GATA(A/G)in downstream targets, and play critical roles in various developmental processes, including cardiac and coronary vasculature development. The transcriptional activity of the GATA transcription factors is modulated through interaction with multiple nuclear proteins, including other zinc-finger proteins such as the FOG family, the NKX2 family, the NFAT family, and coactivators such as p300 and CBP (51, 52) (Fig. 1).

GATA6

Mutations in the gene encoding GATA6 have been reported in familial cardiac outflow tract anomalies including persistent truncus arteriosus (PTA) and PS (53). Mice that are compound heterozygotes for Gata4 and Gata6 null alleles die in utero and exhibit a spectrum of cardiovascular defects, including thin-walled myocardium, ventricular and aortopulmonary septal defects, and abnormal smooth muscle development (54).

NKX2.5

Mutations in the *NKX2.5* (*NKX2E, CSX*) gene cause various CHM, including ASD and VSD, atrioventricular conduction defects, TOF, subvalvular aortic stenosis (AS), pulmonary atresia, Ebstein anomaly, ventricular hypertrophy, cardiomyopathy and ventricular non-compaction (5, 42, 55–63) (Tables 1 and 3). Most *NKX2.5* mutations are

found in familial atrioventricular block with ASD (5, 55, 61) and TOF (62, 63). In other CHM *NKX2.5* mutations are uncommon (63). *Nkx2.5* knockout mice lack the primordium of the AV node (64), whereas ventricular-restricted *Nkx2.5* knockouts display complete heart block and massive trabecular muscle (65). NKX2.5 is a homeobox transcription factor contributing to diverse cardiac developmental pathways through interaction with the network of transcriptional regulators of heart morphogenesis (Fig. 1).

NKX2.6

Only a single mutation in the *NKX2.6* gene has been associated with CHM, in a consanguineous family with PTA (66) (Tables 1 and 3). Targeted disruption of *Nkx2.6* in mice did not result in an abnormal cardiac phenotype (67). NKX2.6 is a homeobox transcription factor with great homology to NKX2.5, but its transcriptional targets are unknown (Fig. 1).

TBX20

Mutations in the *TBX20* gene have been found in a minority (<1%) of CHM patients (7, 68). A missense mutation has been found in a family with autosomal dominant inheritance of septal defects (7). A truncating mutation was present in a family with autosomal dominant inheritance of septal defects, LVOTO anomalies including mild CoA, mitral valve stenosis, hypoplastic left ventricle (HLV) and cardiomyopathy (7) (Tables 1 and 3). Another *TBX20* mutation was reported in a three-generation family with secundum ASD2 and aortic/mitral valve defects (68). Heterozygous *Tbx20* knockout mice show atrial septal abnormalities and dilated cardiomyopathy (DCM), whereas homozygous mutants show a rudimentary heart that lacks chamber myocardium (69). TBX20 is a cardiac T-box factor that interacts with other cardiac transcription factors, including NKX2.5, GATA4, and TBX5 (70) (Fig. 1).

CITED2

Mutations in the *CITED2* gene have been identified in about 1% of sporadic patients with various CHM, including ASD and VSD, and anomalous pulmonary venous return (71) (Tables 1 and 3). *Cited2* −/− embryos die with ASD and VSD, overriding aorta, DORV, PTA, and right-sided aortic arches (72). These mutant mice lack expression of Pitx2c that is a target gene in the Nodal pathway. CITED2 (CBP/p300-interacting transactivator with E/D-rich c-terminal domain, type 2) is a member of the CITED family of cofactors that are

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involved in regulating a wide variety of CBP/p300-dependent transcriptional responses. CITED2 is a transcriptional co-activator of TFAP2 (Fig. 1). One of the TFAP2 transcription factor genes *TFAP2B* is involved in Char syndrome, which is a syndromic CHM characterized by patent ductus arteriosus (PDA) (Fig. 1).

ANKRD1

In a patient with total anomalous pulmonary venous return (TAPVR) showing a de novo t(10;21) balanced translocation, the *ANKRD1* gene was found disrupted (73). An *ANKRD1* missense mutation has been found in another sporadic patient with TAPVR, suggesting that *ANKRD1* gene possibly plays a role in TAPVR (73). The *ANKRD1* gene encodes a transcriptional regulator that belongs to the muscle ankyrin repeat protein (MARP) family.

Contractile protein genes

MYH11

Mutations in the *MYH11* gene encoding the myosin heavy chain 11 are responsible for a specific form of familial thoracic aortic aneurysm and/or dissection (TAAD) with PDA (74) (Tables 1 and 3). Patients with an *MYH11* mutation exhibit a severe decrease in the elasticity of the aortic wall. This is consistent with the role of myosin heavy chain 11 in smooth muscle cells in maintaining the mechanical properties of the thoracic aorta. The perinatal changes of the ductus arteriosus require smooth muscle cells to migrate, proliferate, differentiate, and contract (75). As evidenced by the presence of PDA in these patients and in *Myh11* −/− mice (76), myosin heavy chain 11 is also involved in the perinatal closure of the ductus arteriosus. Myosin heavy chain 11 is a contractile protein that is expressed in smooth muscle cells of the ductus arteriosus and arterial walls (Fig. 1).

MYH6

A single missense mutation in the *MYH6* gene has been found in an autosomal dominant family with ASD (14) (Tables 1 and 3). *MYH6* mutations may also cause a spectrum of phenotypes ranging from DCM to HCM (77). Knockdown expression of Myh6 in chicken prevents atrial septum formation (14). *Myh6* cardiac expression is regulated by the transcription factor Tbx5 in physical interaction with Mef2c (78). Mutations in *TBX5* reduce activation of the *MYH6* promotor and lead to ASD in Holt-Oram syndrome. Similarly, *GATA4*

mutations associated with ASD also affect *MYH6* promotor activation (8) (Fig. 1). In heterozygous mice, ablation of the *Myh6* gene leads to focal fibrotic lesions and cardiac myocyte disarray with impairment of both contractility and relaxation, but no septal defects (79). *MYH6* encodes the alpha-myosin heavy chain, a cardiac sarcomeric protein that is part of the contractile unit of cardiovascular muscle and expressed at high levels in the developing atria.

MYH7

Recently, mutations in the *MYH7* have been shown to cause CHM including Ebstein anomaly and septal defects (16) (Tables 1 and 3). *MYH7* is a cardiac sarcomeric protein gene frequently involved in different forms of cardiomyopathy. Homozygous mutant mice die within a week after birth, while heterozygous mice display hypertrophic cardiomyopathy (HCM), but no CHM (80). *MYH7* encodes the beta-myosin heavy chain, a cardiac sarcomeric protein that is part of the myosin thick filament of cardiovascular muscle.

MYBPC3

Whereas heterozygous mutations in the *MYBPC3* gene are a frequent cause of HCM, compound heterozygosity or homozygosity for truncating mutations in the *MYBPC3* gene not only causes lethal forms of cardiomyopathy, but also septal defects. Several Old Order Amish with lethal HCM, PDA and septal defects (apical muscular VSD, and ASD) have a homozygous truncating mutation in *MYBPC3* (81, 82). Septal defects were also present in neonates with severe HCM due to compound heterozygous truncating mutations (83) (Tables 1 and 3). Transgenic mice with mutant *Mybpc3* exhibit mild ventricular hypertrophy, but no septal defects or other CHM (84). *MYBPC3* encodes a cardiac sarcomeric protein cardiac myosin-binding protein C that modulates myosin, assembly actin−myosin interaction in sarcomeres and stabilizes thick filaments (85).

ACTC1

ACTC1 is another sarcomeric protein gene implicated in HCM, DCM, and non-compaction cardiomyopathy (NCCM). A founder mutation E101K in Spanish families with apical HCM/NCCM also causes secundum ASD or atrial septum aneurism in multiple patients, and VSD in one patient (15). In another family with apical HCM due to a *ACTC1* missense mutation (G99K) one patient also had ASD (86). In two large Swedish families with autosomal dominant inheritance of

ASD without cardiomyopathy a founder mutation M123V was identified (87) (Table 1). However, the frequency of *ACTC1* mutations in ASD overall is low (1–2%), and no *ACTC1* mutations were found in various other types of CHM (87). Actin knockdown in chick embryos produces less-developed atrial septa (87). Mice lacking cardiac actin do not show gross cardiac anomalies, but increased apoptosis in the atrial and ventricular septa (88). Also in the pathogenesis of human secundum ASD apoptosis may play an important role (89). The *ACTC1* gene encodes the cardiac actin protein that is an essential structural component of the thin filaments of sarcomeres. One end of the actin filament forms cross bridges with myosin to generate force, whereas the other end is immobilized and anchored to α-actinin in the Z disc. *ACTC1* mutations in patients with ASD seem to reduce affinity of actin for myosin (87).

Miscellaneous genes

GJA1
Mutations in *GJA1* have been reported in various forms of CHM by the group of Britz-Cunningham (90). However, several other groups were unable to find Cx43 mutations in CHM patients (91–96), and it has been suggested that the mutations identified by the Britz-Cunningham group were not located in *GJA1*, but in the highly homologous *GJA1* pseudogene. Complex mutations indicative of illicit recombination between *GJA1* and the *GJA1* pseudogene have been found in heart tissue from patients with HLHS (97). Cx43-null mice show delayed looping (98). *GJA1* encodes a gap junction protein connexin 43 (Cx43), which facilitates cell-to-cell adhesion and intercellular communication.

FLNA
Four different mutations within the same region (repeat 1–7) of the X-linked *FLNA* gene encoding filamin A have been identified in families affected by valvular dystrophy (99) (Table 1). Loss-of-function *FLNA* mutations are lethal in males, while in females they result in periventricular nodular heterotopia associated with aortic aneurysms, valve regurgitation and overlapping features of Ehlers-Danlos syndrome (100, 101). Mutations that conserve the reading frame lead to a broad range of syndromes, including frontometaphyseal dysplasia, Melnick-Needles syndrome, and otopalatodigital syndrome type 1 (OPD1) and type 2 (OPD2). FLNA-null mice die at midgestation with widespread hemorrhage from abnormal vessels, PTA, and septal defects (102). The *FLNA* gene encodes filamin A, a large cytoplasmatic protein that cross-links actin filaments and participates in the anchoring of the actin cytoskeleton to membrane proteins.

Elastin
A common microdeletion within chromosomal band 7q11.2 encompassing the *ELN* gene causes Williams syndrome, a syndromic CHM with supravalvular AS, poststenotic aortic aneurysms, sometimes associated with arterial stenosis, mainly of pulmonary arteries. Intragenic *ELN* mutations result in the same spectrum of CHM (103, 104) (Table 1). Some patients with *ELN* mutations also show bilateral inguinal hernias, cutis laxa, pulmonary disease, and aortic aneurysm and dissection (105). Transgenic mice hemizygous for the elastin gene show a compensatory increase in the number of elastic lamellae and smooth muscle in their arteries, resulting in arterial stenosis (106). The *ELN* gene encodes elastin that forms the amorphous component of elastic fibers that are abundantly present in arteries.

THRAP2
Mutations in the *THRAP2* gene are present in 3% of patents with non-syndromic TGA (107) (Table 1). The *THRAP2* gene encodes a TRAP240-like protein, which belongs to the TRAP complex of proteins associated with the thyroid hormone receptor.

TLL1
Missense mutations in *TLL1* have been described in patients with ASD, although the significance of these mutations is not clear as only a limited group of 15 healthy controls were screened, no family members were screened for these mutations and no functional analysis of these mutations was performed (108). Mice with a disrupted *Tll1* gene display incomplete formation of the muscular interventricular septum and abnormal positioning of the heart and aorta (109). The *TLL1* gene encodes Tolloid-like-1, an astacin-like metalloprotease that is highly similar to the morphogenetically bone morphogenetic protein-1 (BMP1).

Genes implicated in syndromic CHM

Some genes implicated in syndromic forms of CHM have also been found to cause non-syndromic CHM with no or subtle non-cardiac features. These genes include *JAG1* (Alagille syndrome), *ZIC3* (X-linked heterotaxy), *TBX1* (22q11.2 deletion syndrome), *TBX5* (Holt-Oram syndrome) and

TFAP2β (Char syndrome). Patients with 22q11.2 deletion syndrome or Holt-Oram syndrome might occasionally have family members with only cardiac features or no features at all, although they share the same mutation.

JAG1

JAG1 (JAGGED1) mutations have not only been found in patients with Alagille syndrome, but also in non-syndromic right-sided heart defects such as PS and TOF (110, 111) (Tables 1 and 3). Jagged 1 is a ligand for the NOTCH receptors (Fig. 1). Also *NOTCH2* mutations have been shown to cause Alagille syndrome, but not non-syndromic CHM.

ZIC3

ZIC3 mutations typically result in X-linked heterotaxy, a combination of LR asymmetry defects, including complex cardiac anomalies, altered lung lobation, splenic and hepatobiliary abnormalities, and gut malposition. *ZIC3* mutations have also been found in non-syndromic CHM such as TGA, ASD and PS (112–115) (Tables 1 and 3). *Zic3* mutant mice exhibit heterotaxy, neural tube defects, and vertebral and rib anomalies (116). ZIC3 is a zinc-finger transcription factor that acts as an enhancer of the NODAL signaling pathway (Fig. 1).

TBX1

A common microdeletion within chromosomal band 22q11.2 encompassing the *TBX1* gene causes the velocardiofacial syndrome (22q11.2 syndrome), which is a major cause of CHM. Although intragenic *TBX1* mutations have been shown to cause most of the anomalies of the microdeletion patients (117–119), they can also be associated with non-syndromic CHM, including VSD and IAA (120, 121). *TBX1* encodes a T-box transcription factor which is expressed in neural crest cells (Fig. 1).

TBX5

Whereas *TBX5* null alleles usually lead to classical Holt-Oram syndrome, some patients with a *TBX5* missense mutation (e.g. the G80R mutation) have non-syndromic CHM with very limited limb anomalies, but severe cardiac defects (122, 123) (Table 1). *TBX5* belongs to the Brachyury (T) family, which encodes transcription factors sharing a common DNA-binding motif, the T-box. The TBX5 protein associates with other cardiac transcription factors including GATA4 and NKX2.5, and synergistically activates different cardiac effector target genes (Fig. 1).

TFAP2β

Char syndrome is caused by mutations in the *TFAP2β* gene. Although most *TFAP2B* mutations lead to PDA associated with typical facial dysmorphism, patients with the P62R mutation show PDA with only mild facial features (124, 125) (Table 1). *TFAP2β* is a transcription factor expressed in neural crest cells (Fig. 1).

Reduced-penetrance mutations in susceptibility genes

The hypothesis of the multifactorial inheritance of common diseases suggests that multiple genetic risk factors with reduced penetrance (intermediate or low) superposed on unfavorable environmental factors lead to disease. These risk factors can be rare variants with intermediate penetrance (Table 2) or common gene variants with low penetrance (Table 4).

Rare variants with intermediate penetrance

Rare variants with intermediate penetrance have been associated with CHM, but for most of them there is only limited evidence that they contribute to CHM (Table 2). Most of these mutations are missense mutations in sporadic patients with CHM whose unaffected family members (usually one of the parents) also show this DNA variation; in other cases the mutation is also present in the control population, albeit with a lower frequency. The latter is then considered a circumstantial evidence of low penetrance. However, in many cases the functional significance of such missense mutations is unknown, and they could be either non-functional polymorphisms or disease mutations with reduced penetrance. Additionally, multiple rare variants sometimes can be found in a single patient, implying a cumulative effect and consequently a reduced penetrance for the individual mutations, as shown for the NODAL pathway (10). Most studies involve mutation analysis of the open reading frame of genes already implicated in CHM by the presence of high-penetrance monogenic mutations leading to the respective CHM. As variations can also be located in gene control regions the mutations described below might under-represent the overall amount of intermediate-penetrance mutations.

The different rare variants with intermediate penetrance implicated in non-syndromic CHM are discussed below (Table 2).

Table 4. Common variants with low penetrance contributing to non-syndromic CHM

Gene	Function	Cardiac phenotypes	Low-penetrance mutations[a]	References
MTHFD1	Methylation cycle	TOF, AS	R653Q	(137)
MTRR	Methylation cycle	Various	I22M	(12, 138)
SLC19A1	Methylation cycle	Various	c.80A>G	(139)
NNMT	Methylation cycle	Various	G>A in intron 1[b]	(11)
TCN2	Methylation cycle	Various	P259R	(12)
NPPA	Vasoactive protein	Conotruncal defects	G664A	(140)
NOS3	Vasoactive protein	Conotruncal defects	E298D[c]	(140)
VEGF	Polypeptide mitogen	VSD, PTA, IAA, TOF	−2578C>A, −1154G>A, −634G>C	(142, 144)
NFATC1	Transcription factor	VSD	Duplication of 44 bp in intron 7	(145)

AS, aortic valve stenosis; IAA, interrupted aortic arch; PTA, persistent truncus arteriosus; TOF, tetralogy of Fallot; VSD, ventricular septal defect.
[a]Mutations in the open reading frame are described at the protein level.
[b]Increased risk for CHM when exposed to periconceptional medicines and/or a low dietary nicotinamide intake.
[c]Increased risk for CHM in combination with maternal smoking.

CRELD1

Several *CRELD1* missense mutations have been reported in patients with isolated atrioventricular septal defect (AVSD) (126, 127) and in two patients with Down syndrome, which is the main cause of AVSD (128). These missense mutations were also found in unaffected parents or other family members, indicating incomplete penetrance of these mutations (126, 127) (Table 2). The *CRELD1* gene located on chromosome 3p is also deleted in patients with the 3p-syndrome, which is often associated with CHM, typically AVSD (127, 129). The *CRELD* family of genes encodes for cell adhesion molecules containing cysteine-rich EGF-like domains, which mediate interactions between proteins of diverse function.

NKX2.5

The identification of *NKX2.5* missense mutations in normal parents of children affected with CHM (and in some cases also in healthy controls) indicates that some of these mutations might have reduced penetrance (62, 63) (Table 2).

NOTCH1

In a large series of patients with BAV and/or LVOTO a *NOTCH1* missense mutation with functional significance was showed in almost 7% of patients. Some of these mutations were also present in unaffected parents although echocardiography was only performed in approximately one third of cases. These mutations could therefore represent rare variants with reduced penetrance (22) (Table 2).

GATA4

GATA4 missense mutations in patients with ASD (45) or AVSD (47) were also found in some of their non-affected parents or family members, indicating that some of these mutations might have reduced penetrance (Table 2).

FOG2

A minority of patients with TOF have a mutation in the *FOG2* gene (130). These mutations were inherited from an unaffected parent indicating reduced penetrance. Also patients with chromosomal breakpoints at 8q22, possibly involving *FOG2*, often show TOF (131) (Table 2). Also *Fog2* knockout mouse embryos exhibit TOF (132, 133). FOG2 (Friend of GATA) is a multi-zinc-finger transcription factor modulating the transcriptional activity of GATA4 (Fig. 1).

NODAL

In 10% of Hispanic patients with heterotaxy a G260R mutation in the *NODAL* gene was found, which was shown to exhibit significant impairment of NODAL signaling. As this mutation was also present in one unaffected parent and a control, it must be considered as a rare variant with reduced penetrance (18) (Table 2). Mutations in different genes involved in the NODAL signaling pathway (*NODAL, FOXH1, CFC1* and *GDF1*) co-occur, suggesting a cumulative effect of mutations leading to reduced NODAL signaling (10).

CFC1

The R78W mutation in the *CFC1* gene, which is common in African-American patients with heterotaxy (29) (38), significantly impairs NODAL signaling, but is also found in controls, and must therefore be considered as a rare variant. Also other *CFC1* variants with reduced penetrance have been reported (10, 38, 134) (Table 2).

Patients with a *CFC1* mutation may show a second mutation in other NODAL pathway components, including the *GDF1* and *FOXH1* genes (10). Altogether these findings suggest that rare variants in *CFC1* may represent genetic factors with reduced penetrance for heterotaxy and other CHM.

FOXH1

FOXH1 mutations are among the most common mutations found in CHM (mainly TOF), but several of these patients show a second mutation in the *CFC1* gene, another component of the NODAL pathway, indicating a cumulative effect of mutations leading to reduced NODAL signaling (10) (Table 2).

GDF1

A single patient with undefined CHM has been reported to have both a missense mutation R68H in the *GDF1* gene and a missense mutation F162L in the *CFC1* gene (10) (Table 2). Assuming functional significance of both mutations, this further indicates a cumulative effect of different mutations leading to reduced NODAL signaling.

MYOCD

A single *MYOCD* missense mutation has been found in a patient with PS. This functionally important variant was also present in 0.5% of Hispanic controls (Table 2). Selective ablation of the *Myocd* gene in neural crest-derived smooth muscle cells in mice resulted in PDA (135). *MYOCD* encodes for myocardin, a transcriptional co-activator of SRF that plays a role in myocardial and vascular smooth muscle cell differentiation.

THRAP2

The missense mutation reported in a patient with TGA was also present in the healthy mother of this parent, indicating reduced penetrance (107).

Common variants with low penetrance

Susceptibility genes with low-penetrance mutations (common variants) are being identified at high speed for various common disorders using genome-wide association studies (GWAS). In these studies several hundred thousands of SNPs (single nucleotide polymorphisms) are analyzed simultaneously in large numbers of patients and controls using high-technology platforms. GWAS studies have not yet been performed in CHM. However, small-scale case–control studies have identified common variants which may be associated with

CHM. As the numbers of individuals (both patients and controls) included in most of these studies are limited the conclusions are often tentative; furthermore, many studies are contradictory and have not been replicated. The different common variants with low penetrance implicated in non-syndromic CHM are discussed below (Table 4).

MTHFR

Different enzymes including 5,10-methylenetetrahydrofolate reductase (MTHFR), MTHFD1, methionine synthase reductase (MTRR), SLC19A1 and
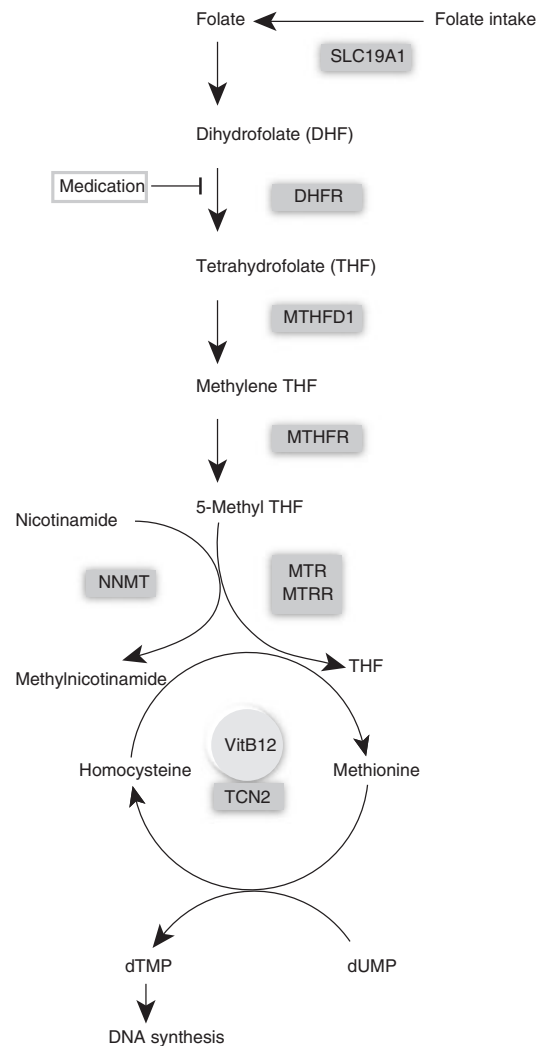

Fig. 2. Methylation cycle with different environmental and genetic risk factors for CHM. Nutritional factors such as reduced folate and vitB12 intake, and common variants in the *SLC19A1, MTHFD1, MTHFR, MTRR, NNMT,* and *TCN2* genes might lead to decreased availability of methionine necessary for DNA synthesis. Folic acid antagonists, including medication such as trimethoprim, triamterene, carbamazepine, phenytoin, phenobarbital, and primidone, may increase the risk for CHM by inhibiting Dihydrofolate reductase (DHFR).

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nicotinamide N-methyltransferase (NNMT) are implicated in the methylation cycle through the conversion of homocysteine into methionine (Fig. 2). MTHFR is an enzyme that converts methylene tetrahydrofolate (THF) into 5-methyl THF. The latter is a cofactor for methionine synthase (MTR) and MTRR, two enzymes that convert homocysteine into methionine. Several studies have reported inconsistent associations between *MTHFR* variants and CHM, but a meta-analysis of published studies concluded that the common *MTHFR* variants A222V (c.677C>T) and E429A (c.1298A>C) on patient or maternal genotypes were not significantly associated with CHM (136).

MTHFD1

The *MTHFD1* gene encodes a trifunctional protein involved in the interconversion of folate to methylene THF. The latter is converted to 5-methyl THF by MTHFR (Fig. 2). The c.1958G>A variant, leading to the missense mutation R653Q in *MTHFD1*, decreases MTHFD1 enzyme stability and activity. Homozygosity for this variant (present in approximately 20% of the European population) is associated with an increased risk for CHM, particularly TOF and AS (137). This effect could only be showed for the R653Q mutation on the patient's genotype, but not on the maternal genotype (Table 4).

MTRR

The *MTRR* gene encodes the MTR activator methionine synthase reductase enzyme. MTRR restores the activity of MTR, the enzyme that converts homocysteine into methionine using 5-methyl THF as a cofactor (Fig. 2). Homozygosity for the I22M variant (c.66A>G) in the mother (138) in combination with low maternal serum vitB12 (12) is associated with an increased risk for different types of CHM in offspring (Table 4).

SLC19A1 (RFC1)

Transport of folate compounds into mammalian cells can occur via receptor-mediated or carrier-mediated mechanisms. One of the genes involved in carrier-mediated transport is the *SLC19A1* gene encoding the reduced folate carrier-1 (Fig. 2). Offspring carrying the G allele for the c.80A>G variation have been reported to show an increased risk for CHM (139) (Table 4).

NNMT

Apart from the *MTHFR*, *MTHFD1, MTRR* and *SLC19A1* genes that are involved in folate metabolism, also the *NNMT* gene may be a genetic determinant of plasma homocysteine levels. NNMT catalyzes the methylation of nicotinamide and other pyridines using methyl groups generated in the methylation cycle of homocysteine-methionine (Fig. 2). A G>A variant in intron 1 in both the maternal and fetal *NNMT* gene are associated with an increased CHM risk, but only on a background of periconceptional exposure to medicines and/or a low dietary nicotinamide intake (11) (Table 4).

TCN2

The *TCN2* gene encodes transcobalamin 2, which is the main transporter of vitB12 (cobalamin), an essential vitamin in the synthesis of methionine (Fig. 2). Maternal and fetal homozygosity for the P259R variant (c.776C>G) in combination with low maternal serum vitB12 have been reported to cause an increased risk for different types of CHM in offspring (12) (Table 4).

NPPA

The *NPPA* gene encodes atrial natriuretic peptide (ANP) that has natriuretic–diuretic activity important in the control of extracellular fluid volume and electrolyte homeostasis. ANP is a cardiac effector hormone secreted from the cardiac atria to decrease blood pressure and cardiac hypertrophy by interaction with different transcription factors and sarcomeric proteins (Fig. 1). The G664A variant in the *NPPA* gene is reported to cause an increased risk for conotruncal defects (140) (Table 4).

NOS3

Endothelial nitric oxide synthase (eNOS) encoded by the *NOS3* gene converts L-arginine into nitric oxide, which plays a role in vasodilatation and in the regulation of cell growth and apoptosis (Fig. 1). Homozygozity for the common c.894G>T (E298D) variation in combination with maternal smoking has been associated with an increased risk of CHM (13) (Table 4).

VEGF

VEGF (vascular endothelial growth factor) is a mitogen that specifically acts on endothelial cells and belongs to a family of regulatory peptides controling blood vessel formation by interacting as a ligand with the endothelial tyrosine kinase receptors FLT1 and KDR/FLK1 (Fig. 1). The AAG haplotype of three variants −2578A, −1154A, and −634G located in the promoter and leader sequence of *VEGF* are known to lower circulating VEGF levels in blood (141). These

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common *VEGF* variants are suggested to confer an increased risk for TOF, both in non-syndromic cases of TOF and syndromic cases with 22q11.2 deletions (142) (Table 4). However, meta-analysis of a recent large study, combining previous studies, showed no support for the hypothesis that common or rare variants in VEGF predispose to CHM, including TOF (143). Newborn mice lacking VEGF die of anomalies reminiscent of 22q11 deletion syndrome with typical cardiac malformations such as TOF (144). *TBX1*, the gene implicated in 22q11 deletion syndrome, is most probably a downstream target of the VEGF pathway as Tbx1 expression is downregulated in these mice.

NFATC1

NFATC1 (nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1) is a calcineurin-dependent transcription factor belonging to the NFAT family of transcription factors. NFATC1 is involved in remodeling of endocardial cushions into mature heart valve leaflets by repression of Vegf expression in the myocardium underlying the site of prospective valve formation (Fig. 1). Two of twenty-one patients with VSD were found to have a homozygous duplication of forty-four nucleotides in intron 7 of the *NFATC1* gene, whereas homozygosity for this duplication was not observed in the control population, suggesting that *NFATC1* is a low-penetrance susceptibility gene for VSD (145) (Table 4).

Chromosomal aberrations

Chromosomal aberrations are well-known causes of syndromic CHM and are detected in 8–13% of children with CHM by conventional cytogenetics alone (146). After the introduction of fluorescence in situ hybridization (FISH) additional deletions in patients with CHM were identified, with the 22q11.2 deletion syndrome as the prime example of a frequent cause of CHM that escaped detection before the introduction of FISH (147). With the recent introduction of array-based comparative genomic hybridization (array CGH) as a routine tool in diagnostics, many more chromosomal regions associated with CHM are being found. A high frequency of chromosomal imbalances was showed in a selected group of patients with syndromic CHM (148). Newly recognized microdeletion/duplication syndromes associated with CHM are the 22q11.1 duplication syndrome (149), the 9q34 deletion syndrome (150), the 17p11.2 deletion syndrome (151), the 16p11.2

deletion syndrome (152, 153) and the 1q21.1 deletion syndrome (154–156). These microdeletions/duplications associated with CHM are good candidate regions to identify CHM genes. Some of the known microdeletion syndromes, such as the 22q11.2 deletion syndrome, can present with CHM without obvious dysmorphic features and/or mental deficit. The recently recognized 1q21.1 microdeletion or -duplication syndrome has an even more variable phenotype with incomplete penetrance (154–156). In a large series of 1q21.1 deletion patients, 25% presented with CHM, including PDA, TA, CoA and BAV (155). This deletion was also found in 3 out of 505 patients with non-syndromic CHM (156). Overall, a high frequency of chromosomal deletions, duplications and copy number variations (CNV) was recently found in two series of non-syndromic patients with CHM (157, 158). These studies indicate that arrayCGH is a powerful tool to identify new loci involved in non-syndromic CHM, and furthermore a useful tool in the diagnostic workup of patients with non-syndromic CHM.

Somatic mutations

Knudson has elegantly showed how somatic mutations not present in the germline can contribute to genetic disease with his two-hit hypothesis (159). There has been much interest in somatic mutations underlying cancer and this has been the subject of many reviews (160). These somatic mutations not only include mutations affecting nuclear DNA leading to activation of oncogenes or inactivation of tumor suppressor genes, but also epigenetic alterations of DNA, and mitochondrial DNA mutations. The concept of somatic mosaicism has also been showed in many different skin disorders, and several other diseases (161). Surprisingly, many years after Knudson's theory gained universal acceptance, the concept of somatic mutations remained largely confined to tumor biology and skin disease. This might explain why the candidate gene approach for many non-syndromic malformations, including CHM, has had little success: mutation analysis in most cases still is usually performed in constitutional DNA, whereas the mutations might be somatic and limited to the affected tissue.

Over the last past years, the first somatic mutations confined to affected cardiovascular tissue have been reported in CHM. The genes in which somatic mutations have been found include *GJA1, NKX2.5, TBX5, GATA4, HEY2* and *HAND1* (162–168) (Table 5). The group of Reamon-Buettner and Borlak has reported

the majority of somatic mutations in CHM, including mutations in *NKX2.5, TBX5, GATA4, HEY2* and *HAND1*. Interestingly, in several patients different mutations in the same gene with cumulative downregulation of transcription were reported (162–168). Although only a limited number of studies have been performed, most probably due to the scarceness of affected cardiac tissue, the abundance of somatic mutations reported by the group of Reamon-Buettner and Borlak contrasts with the limited number of mutations in other studies. No *NKX2.5* gene mutations could be found in cardiac tissue from patients with BAV and associated aneurysm (169), and no somatic 22q11.2 deletions could be identified in heart tissue from patients with conotruncal heart defects without germ line 22q11.2 deletion (170). Recently, no evidence for somatic *NKX2.5* mutations was found in a series of fresh-frozen cardiac tissue taken near the septal defect of patients with ASD, VSD and AVSD (171). The latter authors suggested that the poor DNA quality from the formalin-fixed tissue used by the group of Reamon-Buettner and Borlak may account for the high amount of somatic mutations in their study (171), although differences in the location of tissue sampling might also be important. Evidently, the role of somatic mutations in CHM awaits further confirmation.

The 'multifactorial inheritance' hypothesis

The overall recurrence risk of non-syndromic CHM (defined as the risk of a child with CHM after a previous child with CHM) is usually in the order of 2–10% (172–179). Empirical risk studies have revealed higher recurrence risks for specific subsets of CHM, indicating that genetic factors may play a more prominent role in CHM such as ASD, AVSD, and LVOTO. Several studies have indicated a higher risk for offspring of a mother with CHM than for the children of a father with CHM (9, 180, 181). Theoretically, this could be due to maternal inheritance, imprinting, maternal

environmental factors, but no exact data to explain this discrepancy have been reported.

The 'multifactorial inheritance' hypothesis of common diseases suggests that the interaction and cumulative effect of multiple genetic and environmental risk factors leads to disease. One of the arguments underlying the multifactorial hypothesis is the observation that the overall recurrence risk of non-syndromic CHM is 2–10%; as this is intermediate between the high risk present in monogenic cases and the negligible risk in case of non-genetic CHM, the existence of reduced-penetrance mutations in susceptibility genes has been suggested. However, only a limited number of common gene variants with low penetrance or rare variants with intermediate penetrance have been reported to date (Tables 2 and 4). Furthermore, few interactions of risk factors for CHM have been reported up to now. *MTHFR* polymorphisms might have an effect on heart development when present with other risk factors such as smoking, hyperhomoysteinaemie (182) or nutrient deficiencies (183). Some evidence of an increased risk of conotruncal defects in infants of mothers who smoked cigarettes periconceptional and who had a *NOS3* gene variant has been reported (140). Maternal and fetal variants in the *MTRR* gene (c.66A>G) and in the transcobalamin II gene (c.776C>G) have been reported to be associated with an increased risk for different types of CHM in offspring only in combination with low maternal serum vitamin B12 (12). The A allele in intron 1 of the *NNMT* gene causes an increased CHM risk only on a maternal background of low dietary nicotinamide intake and periconceptional use of certain drugs (11). *VEGF* common variants associated with lower VEGF level confer an increased risk for TOF in patients with 22q11 deletions (142), possibly due to the fact that *TBX1*, the gene implicated in 22q11 deletion syndrome, is a downstream target of VEGF (Fig. 1). Another example of gene–environment interaction is the increase in risk of ASD reported in offspring of

Table 5. Somatic mutations contributing to non-syndromic CHM

Gene	Function	Cardiac phenotypes	Somatic mutations[a]	References
GJA1	Gap junction protein	HLHS	Gene conversion	(97)
NKX2.5	Transcription factor	VSD, ASD, AVSD	Various missense mutations	(165, 166)
GATA4	Transcription factor	VSD, AVSD	Various missense mutations	(198, 199)
TBX5	Transcription factor	ASD, AVSD	Various missense mutations	(164)
HEY2	Transcription factor	AVSD	T96A, D98A, L100S	(163)
HAND1	Transcription factor	HLV, HRV	A126fs	(168)

ASD, atrial septal defect; AVSD, atrioventricular septal defect; HLHS, hypoplastic left heart syndrome; HLV, hypoplastic left ventricle; HRV, hypoplastic right ventricle; VSD, ventricular septal defect.
[a]Mutations in the open reading frame are described at the protein level.

women with low-activity variants of Glutathione-S-transferase when exposed to specific solvents metabolized by Glutathione-S-transferase (184).

The NODAL signaling pathway is a paradigm for multifactorial inheritance of CHM. A minority of patients with heterotaxy (29) or heterotaxy-related HCM such as looping defects (TGA, DORV) (38) or CHM including TOF show several mutations in genes belonging to the NODAL signaling pathway, including the *NODAL, CFC1, FOXH1* and *GDF1* genes (10, 115). As the functional significance of each of these mutations could be showed, the cumulative effects of multiple mutations may lead to reduced NODAL signaling eventually resulting in CHM.

Conclusions

The genetics of non-syndromic CHM remains unclear to a large extent: the low percentage of single gene mutations with high or intermediate penetrance argues against a prominent role of such mutations in non-syndromic CHM, the existence of somatic mutations in CHM heart tissue is still a matter of debate, and it remains uncertain whether common variants have a large contribution. Although more than 40 different genes have already been shown to be implicated in non-syndromic CHM, many human genes are expected to be identified within the coming years, taking into account the large number of genes that have been shown to play a role in cardiogenesis in mice. It is expected that the massive sequencing power of next-generation sequencers will be instrumental in the identification of additional genes implicated in CHM. Furthermore, such studies might shed light on the interaction of different genetic factors, and finally prove or refute the multifactorial model.

Conflict of interest

Both authors declare not to have any conflict of interest regarding the work described here.

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