Tetralogy of Fallot and Hypoplastic Left Heart Syndrome – Complex Clinical Phenotypes Meet Complex Genetic Networks

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Abstract: In many cases congenital heart disease (CHD) is represented by a complex phenotype and an array of several functional and morphological cardiac disorders. These malformations will be briefly summarized in the first part focusing on two severe CHD phenotypes, hypoplastic left heart syndrome (HLHS) and tetralogy of Fallot (TOF). In most cases of CHD the genetic origin remains largely unknown, though the complexity of the clinical picture strongly argues against a dysregulation which can be attributed to a single candidate gene but rather suggests a multifaceted polygenetic origin with elaborate interactions. Consistent with this idea, genome-wide approaches using whole exome sequencing, comparative sequence analysis of multiplex families to identify *de novo* mutations and global technologies to identify single nucleotide polymorphisms, copy number variants, dysregulation of the transcriptome and epigenetic variations have been conducted to obtain information about genetic alterations and potential predispositions possibly linked to the occurrence of a CHD phenotype. In the second part of this review we will summarize and discuss the available literature on identified genetic alterations linked to TOF and HLHS.

## CONGENITAL HEART DISEASE – THE CLINICAL PICTURE

The heart is the first functional organ during embryogenesis. After birth, desaturated blood is received through the right atrium, forwarded to the right ventricle and pumped into the lungs. Oxygenated blood returns through the left atrium and leaves the heart via the left ventricle through the aorta and systemic arteries to supply all organs with oxygen (Fig. **1A**). Unfortunately, approximately 4 to 14 per 1.000 live births are diagnosed with congenital heart disease (CHD) [1, 2] where this well controlled circuit is disturbed. The underlying malformations are manifold ranging from small atrial or ventricular septal defects to highly complex malformations for example an abnormal spatial arrangement of the great arteries resulting in serious hemodynamic changes. As most genome-wide analyses have been conducted almost exclusively in patients with hypoplastic left heart syndrome (HLHS) and tetralogy of Fallot (TOF) we will briefly summarize the major clinical malformations and surgical interventions of those two phenotypes only.

Hypoplastic Left Heart Syndrome (HLHS)

HLHS is characterized by a stenotic or atretic aortic and/or mitral valve, a high-grade hypoplasia of the aorta ascendens and a highly hypoplastic or even completely missing left ventricle (Fig. **1B**). Furthermore, a patent ductus arteriosus must be maintained after birth to achieve sufficient coronary (retrograde via the hypoplastic aorta ascendens) and systemic blood flow. Beside the patent ductus arteriosus an interatrial shunt between the left and right atrium of different sizes ranging from a small foramen ovale to a wide atrial septal defect guarantees shunting of returning pulmonary venous blood from the left to the right atrium. Thereafter the blood is able to pass the tricuspid and pulmonary valve for pulmonary as well as coronary and systemic perfusion via the patent ductus arteriosus.

The HLHS is a rarely occurring CHD, with a prevalence of only 0.15 per 1,000 live births in Germany as reported in the PAN study [2] and the majority of the cases are sporadic. Furthermore, 70% of the affected patients are male, reflecting a strong gender aspect in the disease development [3]. Until recently, HLHS was a uniformly fatal pathologic condition. No definitive treatment existed until in the 1980s the use of the right ventricle to support systemic circulation was first proposed. A tremendous progress in the management of HLHS including prenatal diagnostics, operative procedures

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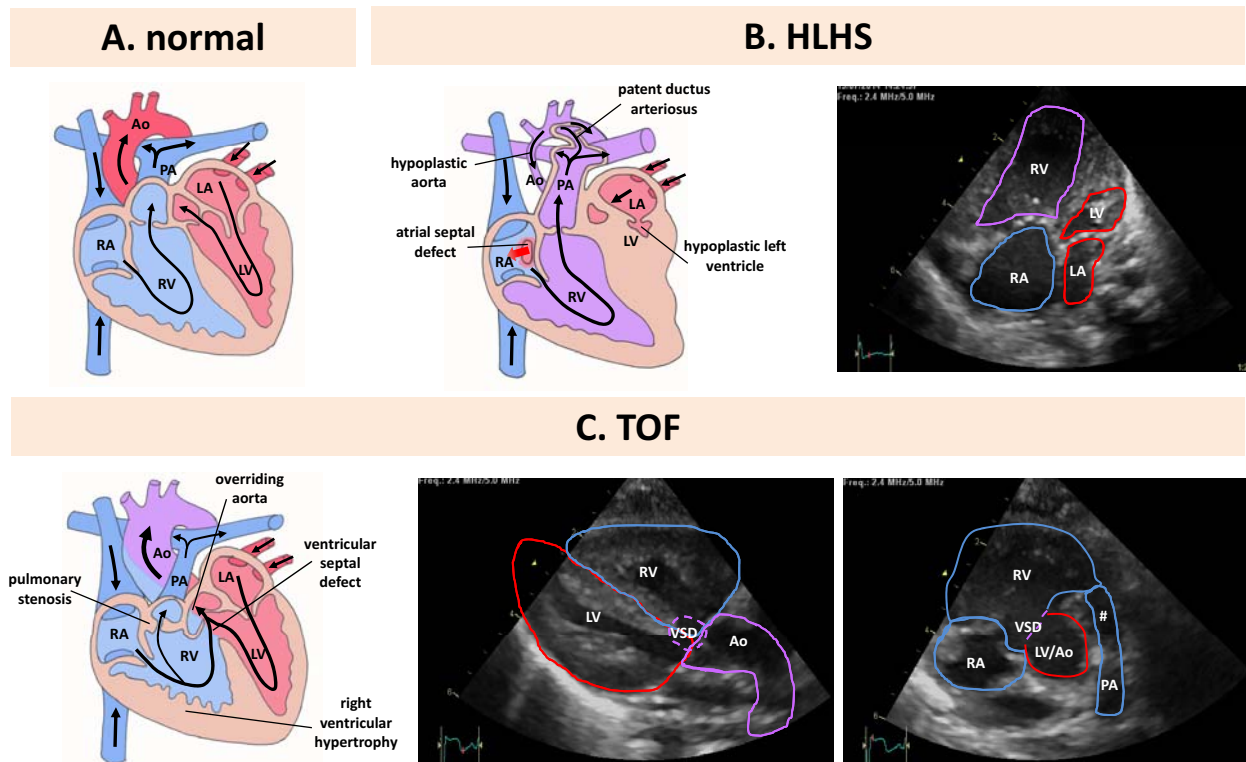
Fig. (1). Comparative illustration of morphology and blood flow in normal heart and CHD. A. Morphology and blood flow in a normal heart. **B. Left panel.** Morphology and blood flow in hypoplastic left heart syndrome. **B. right panel.** Transthoracic echocardiography, apical four chamber view. **C. left panel.** Morphology and blood flow in Tetralogy of Fallot. **C. middle panel.** Transthoracic echocardiography, parasternal long axis view. **C. right panel.** Transthoracic echocardiography, parasternal short axis view. Ao: aorta, LA: left atrium, LV: left ventricle, RA: right atrium, RV: right ventricle, PA: pulmonary artery, VSD: ventricular septal defect, #: narrow right ventricular outflow tract.

and an optimized perioperative intensive care unit treatment has increased patient survival to around 65% at 5- and 55% at 10-years of age [4-6]. At present, the classical surgical strategy encompasses a 3-staged procedure resulting in an univentricular "Fontan" circulation. This palliative treatment ends up with a right ventricle that supports the systemic circulation and a completely passive pulmonary blood flow.

Tetralogy of Fallot (TOF)

One of the most relevant right heart CHDs is TOF first described in 1888 by the French pathologist Étienne-Louis Artur Fallot. TOF has a prevalence of 0.27 per 1.000 live births in Germany [2]. The morphological phenotype is characterized by four malformations: a narrowing of the right outflow tract (pulmonary stenosis), a hypertrophy of the right ventricle, a ventricular septal defect (VSD) and a displacement of the aorta to the right side over the VSD (an overriding aorta) (Fig. **1C**). The TOF is a classical cyanotic CHD due to a subpulmonary obstruction with shunting mainly from the right to the left ventricle via the VSD leading to an ejection of deoxygenated blood into the aorta. At present, surgical repair is performed in the first month of life where the stenosis of the right outflow tract is relieved and the VSD is closed enabling an exclusive ejection of oxygenated blood via the left ventricle. Cumulative survival and event-free survival were 72% and 25%, respectively, after 40 years [7].

CONGENITAL HEART DEFECT – A DISEASE WITH MULTIPLE GENETIC ORIGINS

Severe CHDs are characterized by complex clinical phenotypes with numerous malformations. The multi-factor background of CHD with genetic components was first proposed almost half a century ago [8]. Genetic causes have first been mentioned in the early 80-ies [9] and were confirmed by numerous subsequent epidemiological data [10, 11] and family studies [12-14].

The majority of children born with CHD do not have other physical defects. Nevertheless, it has been noted that major chromosomal abnormalities occur in such children [15, 16]. These obvious chromosomal defects can be detected by conventional karyotyping and G-band analysis and about 10% of CHD in children with multiple congenital defects and/or intellectual disability is caused by trisomies 21, 18 and 13 [17]. However, none of these aberrations can be attributed to a single CHD phenotype and they are associated with different CHDs at variable frequencies (Table **1**). Being the long-time gold standard the resolution of this traditional method is limited to identify chromosomal aberrations of 5 Mb or larger. The frequent 22q11 deletion causing DiGeorge syndrome, a large 3 Mb deletion encompassing some 60 genes, was first discovered by applying quantitative Southern blot hybridization [18].

Table 1. **Aneuploidity and congenital heart defects.**

Chromosomal Aberration	Frequency [a]	Association with Congenital Heart Defects	CHD Frequency in
Trisomy 21 (Down Syndrome)	1 : 700	ASD, AVSD, TOF, VSD	50 %
Trisomy 13 (Patau Syndrome)	1 – 2 : 10.000	ASD, HLHS, PDA, VSD	80 %
Trisomy 18 (Edwards Syndrome)	1 : 2.500	ASD, BAV, CoA, DORV, PDA, TOF, VSD	> 90 %
Monosomy X (Turner Syndrome)	1 : 2.500	AoS, BAV, CoA, HLHS	33 %
47, XXY (Klinefelter Syndrome)	1 – 2 : 1.000	ASD, MVP, PDA	50 %

[a]: per live births. Adapted from [31].

AoS: aortic stenosis, ASD: atrial septal defect, AVSD: atrioventricular septal defect, BAV: bicuspid aortic valve, CoA: coarctation of the aorta, DORV: double-outlet right ventricle, HLHS: hypoplastic left heart syndrome, MVP: mitral valve prolapse, PDA: patent ductus arteriosus, TOF: tetralogy of Fallot, VSD: ventricular septal defect.

Such syndromes, however, appear only in a minor fraction of CHD. Almost 80% of CHDs occur sporadically and their inheritance does not follow a Mendelian order. Meanwhile a large list of potential candidate genes has been identified [19], though these genes have only exceptionally been shown to be causative for the disease such as *TBX5* for the autosomal-dominant inherited Holt-Oram syndrome [20]. Mutations in further essential cardiac transcription factors such as *MESP1* [21], *NKX2.5* [22-24], *GATA4* [25], *GATA6* [26] or *TBX20* [27] and combinations thereof [28] have been described in patients with CHD but without any convincing proof of their causative function.

Therefore, in most cases of CHD the genetic origin still remains largely unknown though the accumulation of alterations in the genome of patients affecting a large number of causative genes appears a plausible explanation. To identify polygenic networks on a genome-wide basis with increased sensitivity, novel technologies have been developed. Array-based comparative genomic hybridization (CGH) techniques allow the detection of copy number changes in the range of 5 to 10 kb [29] or even down to 200 bp [30] and state-of-the-art chips either facilitate the analysis of several millions of SNPs, a genome-wide detection of methylation sites or the global transcriptome evaluation. Next-generation sequencing technologies replace Sanger sequencing and gradually also genomic micorarrays. These high-throughput approaches require a substantial computational and bioinformatic power but yield far more sequence information in less time and at lower costs which makes them the method of choice to analyze multifactorial and multigenetic diseases such as CHD. In the following we will summarize and discuss such genome-wide approaches which have been conducted to identify genetic alterations and potential candidate genes and network-interactions in CHD populations with a defined clinical phenotype.

SINGLE NUCLEOTIDE VARIANTS (TABLE 2)

To identify possible associations of common variants with the etiology of CHD several genome-wide association studies have been initiated. Considering all CHD cases no genome-wide association of a certain risk locus was seen [32]. Therefore, subsequent analyses focused on defined CHD phenotypes.

Tetralogy of Fallot (TOF)

Two recent reports identified genetic variants in the chromosomal region 12q24 as risk factors for TOF [33, 34]. This 1.6 Mb region encompassing 15 genes has previously been reported as a risk factor for several diseases including coronary artery disease [35]. Six different SNPs with strong significance (top SNP rs11065987) were located in this region [33]. The strongest candidate gene is *PTPN11*, a regulator of Ras/MAP-kinase signaling pathway and associated with Noonan syndrome [36]. Most interestingly, the SNP rs11066320 located in intron 6 of the *PTPN11* gene was also reported in the second study which analyzed 22 candidate genes [34]. However, the hypothesis that these SNPs may up-regulate *PTPN11* activity thereby increasing the susceptibility for TOF still remains to be validated. More modest significance levels were seen at 13q32 (rs7982677) in intron 7 of *GPC5* and the non-synonymous substitution Ile733Val in *NRP1* [33]. The latter locus was also confirmed in a Chinese TOF population [37]. Although both genes are involved in cardiac development [38, 39] further studies are necessary to confirm a functional effect of any of these variants.

Grunert and colleagues performed an interesting approach with targeted re-sequencing and whole transcriptome profiling to identify potentially causative genes in TOF [40]. To discriminate causative genes they introduced a gene mutation frequency, calculated by the relation of the number of individuals with deleterious mutations to the total number of individuals with sufficient genotype information. After normalization by the gene-length the method was validated in a retrospective study on a cohort of patients with hypertrophic cardiomyopathy [41]. By this approach they identified deleterious SNPs in 16 TOF genes, six of which have known association with cardiac disease and seven provoke a cardiac phenotype after mutation or knock-out in mice. The vast

Table 2. Genome-wide association studies in CHD patients.

Platform(s)	Patients	Genetic Alteration	Impact	Reference
Illumina 660W-Quad	798 TOF	12q24 (*PTPN11*) 13q32 (*GPC5, NRP1*)	risk allele for TOF risk allele for TOF	[33]
SEQUENOM MALDI-TOF	362 TOF	rs11066320 (*PTPN11*)	risk allele for TOF	[34]
NimbleGen sequence capture 365K array Genome Sequencer FLX Illumina Genome Analyzer	26 TOF	deleterious SNPs in sixteen genes	imbalance of functional networks in TOF	[40]
Applied Biosystems Linkage Mapping Set (medium-density)	43 families with LVOT	2q23, 10q21, 16p12 16p12 10q11 2p15	risk loci for LVOT risk locus for AVS, CoA, HLHS risk locus for AVS, CoA risk locus for HLHS	[42]

majority of mutated alleles could be confirmed by mRNASeq in right ventricle tissue of the respective patient. The search for potential interaction of these TOF genes suggested that an array of genes was assembled in a functional network where the alterations may create an imbalance ultimately leading to the phenotype of TOF. They could indeed confirm this imbalance by analyzing the expression profile in the right ventricle of three TOF patients with mutations in the *MYOM2* gene. A shared differential expression of four TOF genes, including *MYOM2*, was observed. Finally they analyzed right ventricle biopsies which confirmed the expected aberrant histological phenotype.

Hypoplastic Left Heart Syndrome (HLHS)

An analysis of 411 microsatellites focused on the identification of genetic susceptibility loci in multiplex CHD families with left ventricular outflow tract (LVOT) malformations [42]. Three novel regions with suggestive linkage were identified on 2p23, 10q21 and 16p12 by a combined analysis of all included malformations. The analysis of subgroups revealed overlapping linkage signals on 16p12 (aortic valve stenosis (AVS), coarctation of the aorta (CoA), HLHS) and on 10q11 (AVS, CoA). Finally a significant peak on 2p15 restricted to the HLHS cohort was identified. While overlapping peaks strengthen the hypothesis of a common genetic etiology of these malformations, further supported by their combined occurrence in families [43] the data also suggest the presence of distinct alterations potentially involved in the development of specific phenotypes.

COPY NUMBER VARIATIONS (TABLE 3)

The term copy number variation (CNV) is a form of structural variation which becomes evident when an individual genome is compared to the standard reference genome in the database. Certain sections of the DNA exist in different copies due to deletions, insertions, duplications and complex multi-site variants of the genomic DNA. CNVs ranging from 1 kb to several megabases are found in all humans covering approximately 12% of the human genome [44]. Large CNVs have been detected in all major CHD phenotypes [45, 46] and they typically affect half of the cases [47].

Tetralogy of Fallot (TOF)

The first genome-wide study which analyzed CNVs examined 433 unrelated TOF patients. CNVs were detected at a frequency of 63 CNVs per genome with a median size of approximately 18 kb with no major differences between controls and cases [48]. Focusing on rare large CNVs (<0.1% in control population, > 500 kb) revealed an increased prevalence in TOF patients (OR 1.89), most notable in CNVs which overlapped exons (OR 2.54). Among 47 large CNVs the most compelling in TOF patients were duplications in 1q21.1. Pathway analyses identified several candidate genes including *GJA5* thereby confirming previous studies [49, 50]. Most interestingly, the study revealed a novel locus at 1q32.2 in two unrelated TOFs who displayed loss CNVs overlapping the *PLXNA2* gene, an important regulator of endothelial cell function during outflow tract septation [51, 52]. Knockout mice deficient for *Plxna2* display congenital heart defects including TOF [53]. *PLXNA2* is a receptor for semaphorins and two individuals showed a CNV loss in semaphorin genes (*SEMA3D, SEMA3E*). These genes modify the NOTCH pathway [54] and mutations in *SEMA3E* can cause Charge syndrome [55]. Pathway analyses allocated important functions of these genes in vasculature development, cell motility, chemotaxis and neuron projection and development.

The role of 1q21.1, first described as causative for CHD a decade ago [56], was analyzed in more than 948 TOF patients and 1488 patients with other forms of CHD [57]. Duplication of the 1q21.1 locus was more common in TOF patients (OR 30.9) while deletion was more common in other forms of CHD (OR 5.5). Rare 100-200 kb duplications encompassing the *GJA5* gene were strongly enriched in the TOF population (OR 10.7). An altered expression of this gene might possibly contribute to the TOF phenotype as *Gja5*-deficient mice show complex heart defects [58]. The impact of these duplications remains unclear since they were present as *de novo* events and inherited in the patient population.

Hypoplastic Left Heart Syndrome (HLHS)

A large multi-center study, focused on *de novo* CNVs in patients with left-sided CHD including bicuspid aortic valve,

Table 3.   Copy number variations in CHD patients.

Platform	Patients	Genetic Alteration	Impact	Reference
Affymetrix Genome-Wide Human SNP Array 6.0	433 TOF	dup 1q21.1 (*GJA5*) del 1q32.2 (*PLXNA2*)	potential TOF candidate gene potential TOF candidate gene	[48]
Illumina 660W-Quad	948 TOF	dup 1q21.1 including rare 100-200 bp duplications (*GJA5*)	potential TOF candidate gene	[57]
Affymetrix Genome-Wide Human SNP Array 6.0	59 multiplex families with LS-CHD	25 candidate regions (e.g. *CTHRC1*, *MFAP4*, *PLA2G12A*)	potential LS-CHD candidate genes	[59]
NimbleGen high resolution whole-genome array CGH	43 HLHS	multiple del/dup CNVs micro dels in 14q23	association with HLHS, no cardiac candidate genes	[65]

del: deletion, dup: duplication, LS CHD: left-sided CHD.

aortic valve stenosis, coarctation of the aorta and HLHS, analyzed 464 individual genomes including 59 multiplex families and eight trios [59]. Almost 7.000 autosomal CNVs were detected in the patient group (average approximately 15/genome) though these numbers did not differ significantly from the controls. However, 73 CNVs remained unique for the patient group with significant enrichment of genes involved in angiogenesis. Three different approaches were used to more specifically identify candidates harboring an important impact during cardiovascular development: i) *in silico* prediction of gene functionality, ii) screening of SAGE-libraries of embryonic mouse heart libraries for genes with at least three fold higher expression in the outflow tract, and iii) public data base mining. This procedure identified 25 potential candidate genes for left-sided heart disease. These genes include *CTHCR1*, an activator of the Wnt-pathway and *MFAP4*, encoding an extracellular matrix protein involved in cell adhesion and interaction, both highly expressed in developing valves and great vessels [60-62]. Finally, *PLA2G12A*, situated in a locus previously associated with HLHS [63], is an inhibitor of the BMP-pathway [64]. An overlap search with these 25 high priority genes with loci known to be linked to left-sided CHD identified four regions (Xp11.22, 4p16.1, 17p11.2, 1q21) which strengthen the candidacy of the identified genes. In addition, in five different families these unique CNVs segregated with the CHD phenotype.

A small study concentrated on the presence of submicroscopic chromosomal abnormalities in HLHS patients [65]. The frequency of CNVs, predominantly small gains and losses < 60 kb, was increased in the HLHS group (4.60 vs. 2.94 in controls), however, none of them encompassed potential candidate genes for HLHS. Likewise, a unique CNV loss mapping to 14q23.3 which has previously been identified in left heart malformations [63] also does not contain genes associated with cardiac development.

DE NOVO MUTATIONS (TABLE 4)

Several studies have revealed a relatively high number of potentially damaging variations in healthy individuals [66-68] which makes it difficult to identify true disease-related *de novo* events in affected patients. However, the inclusion of first-degree unaffected relatives can minimize this "natural" genetic variation. *De novo* occurring genetic alterations

in an affected patient might be the last event necessary to develop a CHD in combination with a somehow predisposed genetic background, and such events can be identified by comparative analyses of the genetic material of CHD patients and their unaffected parents (a so-called trio).

Hypoplastic Left Heart Syndrome (HLHS)

A first report addressing this question in analyzing HLHS patients used a combined approach of direct sequencing of candidate genes and a genome-wide screening by high resolution array comparative hybridization [69]. In 25 HLHS patients the array CGH analysis identified 33 CNVs. In all cases where the parental DNA could be analyzed, the CNVs were inherited. Two of the identified CNVs (*ZNF423*, *BMPR2*) are associated with cardiac development while the remaining CNVs were located in desert regions or genomic regions without known relevance for cardiac development. Direct sequencing analysis identified several splice site changes, frameshift and missense mutations in *NOTCH1*, *FOXL1* and *FOXC2* which were, however, predicted to be disease causing only for *NOTCH1*. In addition, five variations in regulatory regions of *FOXC2* and *FOXL1* were predicted to be pathogenic. The other two candidate genes (*HAND1*, *NKX2.5*) did not show any genetic lesions.

A large trio collection was investigated for the occurrence of *de novo* and rare CNVs [70]. This study included 148 cases of conotruncal anomalies and 71 cases of HLHS. Despite a rather different pathogenesis the frequency of *de novo* CNVs was comparable in both groups (approximately 10%) but significantly elevated when compared to unaffected controls (approximately 2%). Within the collection of identified *de novo* and ultra-rare CNVs the most significant functional category was 'cardiovascular system development and function' with 15 candidate genes. By applying different criteria (overlapping *de novo* events, identical appearance in affected sibs, affecting known CHD candidate genes, occurrence in more than one trio) the detected numbers of CNVs could be reduced to 19 which are considered to be the most relevant. Three of those candidate genes (*NODAL*, *CFC1*, *NOMO3*) are associated with the Nodal-pathway, underlining its potential role in the development of CHD.

A large study of 362 trios including besides others 84 TOF, 65 TGA, 35 DORV and 60 HLHS patients performed a

Table 4.   *De novo* mutations in CHD patients.

Platform	Patients	Genetic Alteration	Impact	Reference
Agilent customized 400K CGH Array	55 HLHS + 87 parents	del 2q33 (*BMPR2*) dup 16q21 (ZNF423) *NOTCH1* (SS, FS, MS) *FOXC2* (MS), *FOXL1* (MS)	*de novo* mutations with cardiac relevance	[69]
NimbleGen HD2-2.1 CGH	219 Trios (HLHS and conotruncal defects	dup 2q21.2 (*CFC1*) del 10q22.1 (*NODAL*) *de novo* dup 16q13.11 (*NOMO3*)	potential CHD candidate genes	[70]
Illumina 660W SNP Array SNP Array 6.0	2256 CHD 283 TOF families	del 15q11.2 *de novo* dup 1q21.1 (*GJA5*) *de novo* del 4q34 (*HAND2*) *de novo* dup 5q14.2 (*EDIL3*) *de novo* dup 5q35.3 (*CNOT6*)	risc locus for sporadic CHD potential TOF candidate gene potential TOF candidate gene potential TOF candidate gene potential TOF candidate gene	[50]
Affymetrix Human Genome-Wide SNP Array 6.0	114 TOF Trios 398 TOF	*de novo* dup 1q21.1 (*PRKAB2, CHDIL, BCL9, GJA5*)	potential TOF candidate genes	[49]
Illumina HiSeq2000 Sequencer	362 CHD Trios	*de novo* mutations (*MLL2, WDR5, CHD7, KDM5A, KDM5B, UBE2B, RNF20, USP44, SMAD2*)	potential role of chromatin modeling in CHD cardiac candidate genes	[71]

SS: splice-site mutation, FS: frame-shift mutation, MS: missense mutation, del: deletion, dup: duplication.

whole-exome sequencing approach to identify *de novo* mutations in CHD patients [71]. Their results indicate that *de novo* mutations contribute to approximately 10% of severe CHDs. They hypothesized that genes which contribute to CHD should be expressed in the developing cardiac tissue. Analyzing the top 25% genes of the transcriptome in murine E14.5 hearts (defined as high heart expression, HHE) they identified a significantly increased rate of protein-altering *de novo* mutations in HHE human ortholog genes in CHD patients (OR 2.53). Focusing on damaging mutations such as premature termination, splice-sites or frame-shifts increased the odds ratios further to 7.50. In contrast, no significant difference in the mutation frequency between CHD and controls was seen in genes with low heart expression (LHE, 75% of the genes). A closer examination of the mutated gene set revealed an enrichment of eight genes involved in the H3K4 network, a mark for both positive and negative gene regulation including key players of embryonic developmental and cardiogenesis [72-74]. Of note, the phenotype of the patients harboring these mutations was quite diverse. Interestingly, a *de novo* mutation of *SMAD2* which also affects the H3K27 methylation pathway was identified twice, a finding unlikely to occur by chance. These results attribute a potential common role to H3K4 methylation as an important regulator of CHD pathogenesis though it could not be assigned to a defined pathogenic phenotype.

Tetralogy of Fallot (TOF)

More than 2.200 CHD patients including 283 TOF families were analyzed for the appearance of CNVs [50]. They report on overall significantly increased (1.8-fold) risk for genic CNVs (defined as CNVs overlapping with RefSeq boundaries), even further pronounced when considering only deletions of > 1Mb (4-fold increase) with no apparent difference between TOF and other CHDs. The number of genes spanned by either deletions or duplications was consistently higher in patients compared to controls. An annotation enrichment analysis on rare deletions showed a statistically significant 2.9-fold enrichment of genes in the Wnt-signaling pathway comprising 13 different genes across all investigated CHD phenotypes. In addition, a 15q11.2 deletion was the strongest indicator for the risk of sporadic non-syndromic CHD. TOF cases showed an almost 2-fold increase of genic duplications which was, however, solely due to a single locus (1q21.1) encoding *GJA5*, a potential candidate gene for the CHD phenotype [57, 58]. However, this conclusion could not be supported by others [59, 70]. In contrast, a Pro265Ser mutation in *GJA5* (Connexin 40) was detected in TOF patients. This mutation prevents gap-junction formation and disrupts the morphology of the heart tube upon injection into zebrafish [75]. In addition, further rare de novo CNVs affecting genes with cardiac importance (*HAND2, EDIL3, CNOT6*) were identified in a small fraction (approximately 5%) of the TOF population.

A trio analysis of 114 children with TOF and their unaffected parents identified 11 rare *de novo* CNVs at 10 distinct chromosomal loci [49]. Of interest, the 1q.21.1 locus was affected in multiple cases and four individuals shared a duplicated region encompassing four genes (*PRKAB2, CHD1L, BCL9, GJA5*) with the highest expression in the right ventricular outflow tract which is malformed in TOF. However, the authors clearly state that none of the studies performed including their own could show a perfect correlation between 1q21 dosage and the CHD phenotype so far.

ANALYSIS OF GENE EXPRESSION (TABLE 5)

Tetralogy of Fallot (TOF)

The analysis of genome-wide expression in tissue of CHD patients started about a decade ago [76]. In this study

Table 5.   Gene expression analyses in CHD patients.

Platform	Patients	Changes in Expression	Impact	Reference
Human Unigene Set-RZPD2 cDNA Array	55 CHD	88 dysregulated genes (e.g. *SNIP, A2B1, KIAA1437*)	potential relevance for TOF	[76]
Applied Microarrays Inc. CodeLink Human Whole Genome Bioarray	19 TOF	1062 dysregulated genes (e.g. WNT and NOTCH pathway)	potential relevance for TOF	[81]
Affymetrix Human Exon 1.0 ST v2	16 TOF	*NEFH, SST, TF, KRT6A, KRT7, NTRK2, MYL2, PPBP, NRGN, APOC3*	dysregulated network in TOF	[84]
Affymetrix Human Exon 1.0 ST	6 HLHS	153 and 96 dysregulated genes vs. normal RV and LV, respectively	potential relevance for HLHS	[87]
Affymetrix GeneChip microRNA 1.0	24 TOF	61 dysregulated miRNAs	dysregulation of 44 cardiac genes in TOF	[86]

RV: right ventricle, LV: left ventricle.

the expression profiles of the right ventricle obtained from different CHD patients were compared to corresponding normal tissue from donors without cardiac disease. The authors reported a characteristic expression signature of 88 genes in the right ventricle of TOF patients. Interestingly, this TOF-specific signature was different from tissue of right ventricle hypertrophy. Most characteristic was an up-regulation of several ribosomal proteins. In addition, the dysregulation affected several pathways which are involved in cardiac development. Genes such as *SNIP* and *A2BP1* affect central pathways essential for normal cardiac development [77, 78] or play a role in heart embryogenesis [79]. Finally, they have identified a dysregulation of *KIAA1437*which directly binds K-RAS whose deletion leads to cardiac malformations in mice [80].

A second study included both idiopathic TOF patients and three which harbor the syndromic 22q11.2 deletion [81]. More than 1.000 dysregulated genes were identified in the TOF-derived right ventricles which were associated with protein synthesis, cardiovascular disease, genetic disorder, neurological disease and cell death as the top five networks. The expression of the majority of genes associated with the WNT and NOTCH-signal pathway were significantly reduced, suggesting a mutational convergence leading to general gene suppression of these pathways within the right ventricle. Their results also confirmed an up-regulated expression of *VEGF* and proteins of the extracellular matrix reported in earlier studies [82]. A significant up-regulation of angiogenic factors including *VEGF* was also seen by Peters and colleagues who analyzed primary corrected TOF in infants and adult patients who underwent a second corrective surgery for pulmonary regurgitation [83]. The expression of the genes located in the 22q11.2 region was half-reduced in syndromic patients as expected while none of these genes was differentially expressed in any of the idiopathic TOF subjects. Most interestingly, the NOTCH pathway was also suppressed in patients with 22q11.2 deletions [81]. The comparison of idiopathic and syndromic patients strongly suggests that different genetic starting conditions may nevertheless lead to a common pathological outcome.

A different approach to screen for altered gene expression in TOF patients was used by Rodemeyer and colleagues [84]. They started with a panel of 56 genes, a so-called Gene Expression Template (GET), which predicts 24 distinct human tissues under disease-free conditions with very high accuracy [85]. They extracted expression levels of 54 of these genes from previous work [86] and were able to combine the TOF samples in an independent cluster compared to several control tissues. Thirty-three of the analyzed gene showed a significantly altered expression compared to normally developing right ventricles. Nineteen GET genes were significantly altered in TOF compared to normal infant right ventricle. Of these, ten genes were associated with cardiac development, myogenesis and cardiac hypertrophy. Thus, applying the GET signature it was possible to discriminate pathologic TOF tissue from matched normally developing right ventricles.

Hypoplastic Left Heart Syndrome (HLHS)

Recently the first report of a genome-wide expression analysis in tissue derived from HLHS patients was published [87]. They collected right ventricle samples from HLHS patients and right and left ventricles from control neonates. Compared to left and right control ventricles 153 and 96 genes, respectively, were differentially expressed in pathologic tissue with a 66 gene overlap between both comparison groups. The expression profile of HLHS tissues remained constantly different from controls, irrespective of postnatal age. Looking at the top ten candidate genes which showed differential regulation revealed no obvious association with cardiac networks and none of these genes was located near chromosomal regions which have previously been associated with hereditary HLHS [63]. However, the most significantly up-regulated pathway was oxidative phosphorylation suggesting a shift from normal fatty acid towards glucose metabolism, characteristic for myocytes undergoing hypertrophy [88, 89]. Alternative splicing represents an important mechanism to regulate specific gene expression in tissues, including the myocardium [90]. Alternatively spliced mRNAs have been linked to the pathogenesis of certain types of CHD [91, 92] and they have been shown to affect

key sarcomere genes in cardiomyopathies and AoS [93]. In HLHS tissue exon array analysis identified almost 1.500 genes which underwent alternative splicing events, significantly influencing the 'spliceosome' and 'arrhythmogenic right ventricle cardiomyopathy' KEGG pathways [87]. Thus, HLHS-derived tissue is characterized by distinct expression and splicing signatures consistent with the pathophysiological changes in the right ventricle. However, it should be noted at this point that this study describes alterations which appear as a consequence of the altered hemodynamics in HLHS while the genetic defect, preventing the normal development of left heart structures, occurs much earlier.

Non-coding RNAs

Non-coding RNAs such as microRNAs (miRNAs) have been associated with the development of normal heart and they can modulate cardiovascular disease [94-97]. In TOF samples the expression of 61 miRNAs was significantly changed while none of the miRNAs previously correlated with normal heart development showed an altered gene expression [86]. Noteworthy, the expression of miR-1 and miR-133 which have already been linked to cardiovascular disease by different groups [98-100] remained unchanged in the TOF samples. The top five candidates which showed the largest change in expression between TOF samples and normal tissue were validated by qRT-PCR in all samples used for the array analysis and this result was confirmed in additional 8 independent TOF specimens. Analyzing putative target genes of the 61 miRNAs revealed a clear enrichment in networks associated with cardiac development. Comparing the expression of the 61 miRNAs with expression data obtained from exon arrays performed on the same TOF samples revealed a significant change in expression in 173 genes of these cardiac networks and about 25% of them showed a clear-cut inverse correlation with one or more of the 61 miRNAs. This first analysis of non-coding RNAs in CHD impressively shows the tremendous regulatory changes in the myocardium by such molecules.

DNA METHYLATION (TABLE 6)

DNA methylation is a major mechanism of the cell to reversibly regulate gene expression without altering the nucleotide sequence. It involves covalent addition of a methyl-group to CpG dinucleotides, mostly located in CpG islands of the promoter regions but may also occur in regions of lower CpG density, so-called CpG island shores [101]. Genome-wide methylation profiling of murine embryonic hearts between E11.5 and E14.5 has shown a mostly stable global methylome with a minority of differentially methylated sites. These sites are strongly enriched in genes involved in heart development and affect the expression of cardiac-specific genes such as *Has2* required for heart valve development [102].

Importantly, it appears clear that specific methylation patterns can also be inherited [102]. To address potential transgenerational effects of DNA methylation a genome-wide approach analyzed more than 27.000 CpG sites primarily located in the promoter region of more than 14.000 genes in genomic DNA from blood samples drawn after gestation- from 180 mothers with non-syndromic CHD pregnancies [104]. The cardiac phenotypes included atrial or ventricular

defects (47.2%), right- and left-sided obstructed defects (35.4%) and conotruncal defects (16.7%). Compared to the pattern of mothers with unaffected pregnancies 425 sites showed a differential methylation. The vast majority of the sites was located in CpG islands and almost 90% were hypermethylated. The affected genes were strongly enriched in processes affecting fetal development, some of which may influence the development of CHD (e.g. nucleic acid metabolism, signal transduction or anatomical structure development). In addition, a gene set enrichment analysis revealed a functional overlap in pathways involved in embryonic heart development. Several differentially methylated genes have previously been implicated in heart development such as *EGFR* [105], *GATA4* [106, 107] or *Wnt5a* [108]. However, some limitations of this study should be mentioned as the blood samples were drawn after the pregnancy and there is a lack of data on the methylation status of the infants. Nevertheless, these data strongly suggest an impact of the maternal methylation status for the risk of CHD.

Tetralogy of Fallot (TOF)

Several reports analyzing right ventricular myocardium tissue in collectives of TOF patients have shown an increased methylation status in essential cardiac genes such as *NKX2.5* and *HAND1* which was also reflected in decreased gene expression [109, 110]. The same group performed an extended study which included the promoter regions of 71 CHD candidate genes which have previously been shown to be differentially methylated [104]. Their results indicated an up-regulated methylation in 17 genes including the abovementioned *NKX2.5, HAND1* and *HAS2* (essential for heart valve development, [102]) while nine genes were hypomethylated [111]. Based on their role as key players during heart development, seven CHD candidate genes were further analyzed. Their mRNA expression was clearly down-regulated and the simultaneous hypermethylation of *EGFR*, *EVC2* and *NFATC2* was suggested as a representative CpG island methylator phenotype during TOF development [111].

The highly repetitive long interspersed nucleotide element-1 (LINE-1) encompasses up to 25% of the human genome [112]. It is most heavily methylated in its 5'-UTR and can serve as a surrogate marker of global genomic DNA methylation [113, 114]. Applying this marker to mothers who delivered singleton non-syndromic CHD births showed a reduced global methylation status with an almost doubled risk for CHD in the lowest decile [113]. Using the same approach a significantly lower methylation of LINE-1 was detected in TOF patients and those in the lowest quartile had a significantly increased risk for TOF [109]. In good agreement with these data are further results which confirm a decreased expression of DNA methyltransferase in TOF patients [115].

At a first glance it appears contradictory how both hyper- and hypomethylative events may contribute to the development of the CHD phenotype. However, hypomethylation may lead to deleterious changes in gene expression [113], possibly as a consequence of altered binding affinities for transcription factors [116]. In addition, hypomethylation of repetitive elements may generate an overall genomic instability which may be inherited to the progeny [117]. Together

Table 6.   Alteration of DNA methylation in CHD patients.

Platform	Patients	Changes in Methylation	Impact	Reference
Illumina Infinium Human Methylation27 Bead Chip	180 mothers with non-syndromic CHD pregnancy	425 sites with differential methylation (e.g. *EGFR*, *GATA4*, *WNT5S*)	risk for CHD pregnancy	[104]
Sequenom MassArray EpiTyper	30 TOF	hypermethylation of *NKX2.5* and *HAND1*	potential relevance for TOF	[110]
Sequenom MassArray EpiTyper	32 TOF	hypermethylation of *NKX2.5* and *HAND1*, hypomethylation of *TBX2* and *LINE-1*	potential relevance for TOF increased risk for TOF	[109]
Sequenom MassArray EpiTyper	42 TOF	17 genes hypermethylated, 9 genes hypomethylated	representative methylator phenotype phenotype during TOF development	[111]
MethyLight	180 mothers with non-syndromic CHD pregnancy	global hypomethylation (*LINE-1*)	risk for CHD pregnancy	[113]

with a decreased expression of central cardiac transcription factors [109-111] this may lead to a scenario which increases the risk to develop CHD.

INTERACTIONS BETWEEN THE ENVIRONMENT AND THE GENOME

It has become clear that the environment can have a major influence on human development. Factors such as exposure to environmental air pollution may affect up to 25% of human disorders and may be responsible for a 36% death rate in children up to the age of 14 years [118]. However, except for some particular agents such as radon [119] or asbestos [120] it is difficult to establish a direct connection between an environmental influence on an individual disease. The abnormal formation of heart or the great vessels occurs during the first two months of pregnancy and, therefore, the first trimester of pregnancy is the most crucial period of maternal exposure to environmental pollution in CHD. Parental exposure to pesticides has been associated with the occurrence of different congenital heart disorders [121, 122]. In particular, herbicides and rodenticides have been associated with a transposition of the great arteries [123]. In addition, occupational exposure of the mother to chemicals led to an increased risk of ventricular septal and conotruncal defects [121, 124]. Furthermore, air pollutants such as carbon monoxide may increase the risk of TOF, especially during the first two months of gestation [125]. However, in many cases the validity of these observations is weakened by the low number of reported cases and the lack of an appropriate control group. Other factors which influence the developing embryo come from the mother itself. Smoking especially during the first trimester appears to increase the risk to develop CHD, e.g. ASD, pulmonary valve and artery anomalies [126-128]. A clear risk factor for birth defects including CHD is pregestational maternal diabetes mellitus [129, 130] which may account for more than 2.600

CHD cases in the US per year including TOF (230 cases) and HLHS (75 cases) [131].

In a zebrafish model the effect of exposure to retinoic acid and a dioxin-derivative transcriptional genome-wide profiles were analyzed. Quite rapidly a cluster of cell cycle regulatory genes was dramatically down-regulated together with a massive upregulation of *Nr2F5*, a transcriptional repressor, and a cessation of heart growth [132]. In humans some information is available concerning the target genes affected by environmental influence. A set of "environmental target genes" (direct targets of teratogens) and "environmental responders" (genes with altered cardiac expression in model organisms with human monogenic CHD mutations) has been defined after compiling datasets from studies of CHD in humans and in animal model organisms [133]. A set of seven environmental target genes was identified. These include several retinoic receptors, aryl hydrocarbon receptors and a folate reductase. The retinoic acid receptors appear to be risk factors for the outflow tract, while *TBX5* was identified as a risk target or responder gene for the outflow tract and atrial and ventricular septum [133]. The *JAG1* genes which appeared in multiple datasets has previously been associated with TOF [134]. Finally this multi-dataset analysis revealed similar functional networks for CHD risk which include the NOTCH, BMP, TGFβ and PDGFR signaling pathway [133]. Taken together these data suggest that functional interactions between the variation of the genome and the exposure to external environmental factors are rather complex.

SUMMARY

Within the past years the knowledge on the genetics of CHD has tremendously increased though at the same time this information has confirmed the complexity of the etiology. At present our understanding of the genetic reasons strongly suggests a polygenic genetic network and multifaceted interactions. On the genome level these components

Table 7.   List of genes with potential relevance for TOF and HLHS.

Gene	Chromosome	OMIM	Cellular Function	Related Human Diseases and Known Cardiac Phenotypes	Reference
Tetrralogy of Fallot					
BARX1	9q22.32	603260	transcription factor		[40]
FOXK1	7p22.1	no entry	transcription factor		[40]
HAND2	4q34.1	602407	transcription factor		[50]
CNOT6	5q35.3	608951	transcriptional regulation		[50]
A2BP1	16p13.3	605104	transcriptional regulation		[76, 81]
TCEB3	1p36.1	600786	transcriptional regulation		[40]
PTPN11	12q24	176876	modulation of Ras/MAPK signaling	Noonan syndrome, Leopard syndrome	[33, 34]
GPC5	13q32	602446	modulation of Wnt, Hedgehog, FGF, and BMP signaling	acquired nephrotic syndrome, Hurler syndrome, Hunter syndrome	[33]
NRP1	10p12	602069	modulation of VEGF signaling	Kallmann syndrome	[33]
SEMA3E	7q21.11	608166	modulation of NOTCH pathway	CHARGE syndrome	[48]
BCL9	1q21.2	602597	modulation of Wnt signaling	B cell malignancies	[49]
TP53BP2	1q41	602143	cell communication		[40]
WBSCR16	7q11.23	no entry	cell communication	Williams Beuren syndrome	[40]
SNIP	17q12	610786	cell communication		[76]
BRDG1	4q13.2	604298	cell communication		[76]
VEGF	6p21.1	192240	cell communication	Susceptibility to atherosclerosis and diabetic retinopathy	[81, 82]
NPPA	1p36.21	108780	cell communication	atrial fibrillation, atrial standstill	[81]
NPPB	1p36.22	600295	cell communication		[81]
SST	3q27.3	182450	cell communication		[84]
PPBP	4q13,3	121010	cell communication		[84]
NRGN	11q24.2	602350	cell communication		[84]
MYOM2	8p23.3	603509	cell growth and maintenance		[40]
EDIL3	5q14	606018	cell growth and maintenance	Ankylosing spondylitis	[50]
TEKT2	1p34.3	608953	cell growth and/or maintenance		[76]
MYL2	12q24.11	160781	cell growth and/or maintenance	familial hypertrophic cardiomyopathy	[84]
NEFH	22q12.2	162230	cell growth and/or maintenance	amyotrophic lateral sclerosis	[84]
KRT6A	12q13.3	148041	cell growth and/or maintenance	Pachyonychia congenita	[84]
KRT7	12q13.3	148059	cell growth and/or maintenance		[84]
BCCIP	10q26.1	611883	cell cycle control		[40]
DAG1	3p21.31	128239	cell adhesion	Muscular dystrophy	[40]
KIAA1437	9q34.11	608360	cell adhesion	Agammaglobulinemia	[76]
GJA5	1q21.1	121013	gap junction	atrial fibrillation	[48, 49], [50, 57]
ROCK1	18q11.1	601702	Ser/Thr kinase		[40]

Gene	Chromosome	OMIM	Cellular Function	Related Human Diseases and Known Cardiac Phenotypes	Reference
Tetrralogy of Fallot					
STK33	11p15.3	607670	Ser/Thr kinase		[76]
NTRK2	9q22.1	600456	Tyr kinase	hyperphagia, and developmental delay	[84]
PRKAB2	1q21.1	602741	AMP-activated protein kinase subunit		[49]
PEX6	6p21.1	601498	ATPase activity	Peroxisome biogenesis disorder	[40]
FANCL	2p16.1	608111	ligase activity	Fanconi anemia	[40]
FANCM	14q21.1	609644	DNA repair	Fanconi anemia	[40]
CHD1L	1q21.1	613039	DNA repair		[49]
PLXNA2	1q32.2	601054	axon pathfinding	cardiac hypertrophy	[48]
SEMA3D	7q21.11	609907	development of peripheral axons	Hirschsprung disease	[48]
TTN	2q31.2	188840	muscle development	Dilated cardiomyopathy, hereditary myopathy, tibial muscular dystrophy	[40]
EDN1	6p24.1	131240	vasoconstriction	Auriculocondylar syndrome	[40]
HCN2	19p13.3	602781	ion channel	Sinus dysrhytmia	[40]
FMR1	Xq27.3	309550	mRNA trafficing	Fragile X mental retardation, syndrome, ataxia syndrome	[40]
APOC3	11q23.3	107720	transport activity	Apolipoprotein C-III deficiency	[84]
TF	3q22.1	190000	transport activity	Atransferrinemia	[84]
Hypoplastic left heart syndrome					
FOXC2	16q24.1	602402	transcription factor	Lymphedema-distichiasis syndrome	[69]
FOXL1	16q24.1	603252	transcription factorr		[69]
ZNF423	16q12	604557	transcription factor	Joubert syndrome, Nephronophthisis	[69]
CHD7	8q12.2	608892	transcriptional regulation	CHARGE syndrome	[71]
MXD1	2p13.3	600021	transcriptional regulation		[87]
KDM5B	1q32.1	605393	transcriptional regulation		[71]
CTHRC1	8q22.3	610635	modulation of Wnt pathway	HELLP syndrome	[59]
PLA2G12A	4q25	611652	modulation of BMP pathway		[59]
BMPR2	2q32-33	600799	modulation of BMP and TGFβ signaling	Pulmonary hypertension	[69]
NOTCH1	9q34.3	190198	modulation of NOTCH signaling	Aortic valve disease, Adams-Oliver syndrome	[69]
NODAL	10q22.1	601265	modulation of NODAL pathway	Heterotaxy, double-outlet right ventricle, transposition of the great arteries	[70]
NOMO3	16p13	609159	modulation of NODAL pathway	Heterotaxy	[70]
SMAD2	18q21.1	601366	modulation of TGFβ signaling	Dextrocardia	[71]
ANK1	8p11.1	612641	cell communication	Spherocytosis	[87]
PPFIA2	12q21.31	603143	cell communication		[87]

(Table 7) contd….

| Gene	Chromosome	OMIM	Cellular Function	Related Human Diseases and Known Cardiac Phenotypes	Reference
Hypoplastic left heart syndrome					
PMP22	17p12	601097	cell communication	Charcot-Marie-Tooth disease, Dejerine-Sottas disease, Neuropathy, Roussy-Levy syndrome	[87]
SPTA1	1q32.1	182860	cell growth and/or maintenance	Elliptocytosis, Pyropoikilocytosis, Spherocytosis	[87]
CCNJ	10q23.33	no entry	cell cycle control		[87]
MFAP4	17p11.2	600596	cell adhesion and interaction	Smith Magenis syndrome	[59]
MLL2	12q13.12	602113	histone methyltransferase	Kabuki syndrome	[71]
WDR5	9q34	609012	subunit of histone-methyltransferase complex		[71]
KDM5A	12p13.33	180202	histone demethylation		[71]
RNF20	9q31.1	607699	ubiquitination and methylation		[71]
USP44	12q22	610993	ubiquitination		[71]
UBE2B	5q31.1	179095	DNA repair		[71]
GYPB	4q31.21	111740	immune response	susceptibility to malaria	[87]
PLD6	17p11.2	no entry	hydrolase activity		[87]
FUT8	14q24.3	602589	fucose transfer		[65]
CFC1	2q21.1	605194	patterning of right-left embryonic axis		[70]
GRM1	6q24.3	604473	transport activity	autosomal recessive spinocerebellar ataxia	[87]
CACNB2	10p12.31-33	600003	transport activity	Brugada syndrome	[87]

include single base-pair variants (SNPs, point and *de novo* mutations), duplications or deletions of larger sequences (CNVs, InDels) as well as aberrant methylation patterns. On the transcriptome level key players of important developmental and cardiac pathways but also non-coding RNA species are abnormally expressed. An overview of candidate genes with potential relevance for TOF and HLHS is given in (Table **7**). The entirety of these alterations and their interaction results in a complex "CHD genotype" ultimately leading to the development of a distinct CHD phenotype (Fig. **2**). In addition, hemodynamic changes and environmental influences may complete the complicated picture.

A number of distinct candidate genes have been pinpointed and several genetic risk factors with high significance have been identified, though at present no subsequent downstream experiments exist which substantiate the functional relevance of any of these factors. It has not been possible yet to define a well-defined set of genetic alterations which will reproducibly generate a complex CHD phenotype such as TOF or HLHS in an animal model. Furthermore, alterations in non-coding regions such as promoter or enhancer regions may be involved but such information is only scarcely available [135]. Whole genome sequencing approaches can yield results in that field but appropriate studies have not been conducted yet.

Currently the genetic information on severe CHD phenotypes extracted from genome-wide approaches is almost exclusively restricted to TOF and HLHS. Patients with other CHD subtypes, e.g. double-outlet right ventricle or transposition of the great arteries appear in several of the investigated cohorts as smaller subgroups. However, in all instances specific genetic lesions have not been assigned to those subtypes and their numbers were too low to draw any significant conclusions with regard to a potential relevance of genetic alterations.

Statistically valid data can only be obtained by large-scale multicenter studies. Therefore, several network studies have been launched such as the Pediatric Cardiac Genomics Consortium [136], the Deciphering Developmental Disorders study [137] or the UK10K project [138] to analyze the complex genetics of CHD. High-throughput technologies and next-generation sequencing approaches with high numbers of patients and their parents should shed some light on the still mostly enigmatic components involved in genetic predisposition. It will also be challenging to detect potential loci
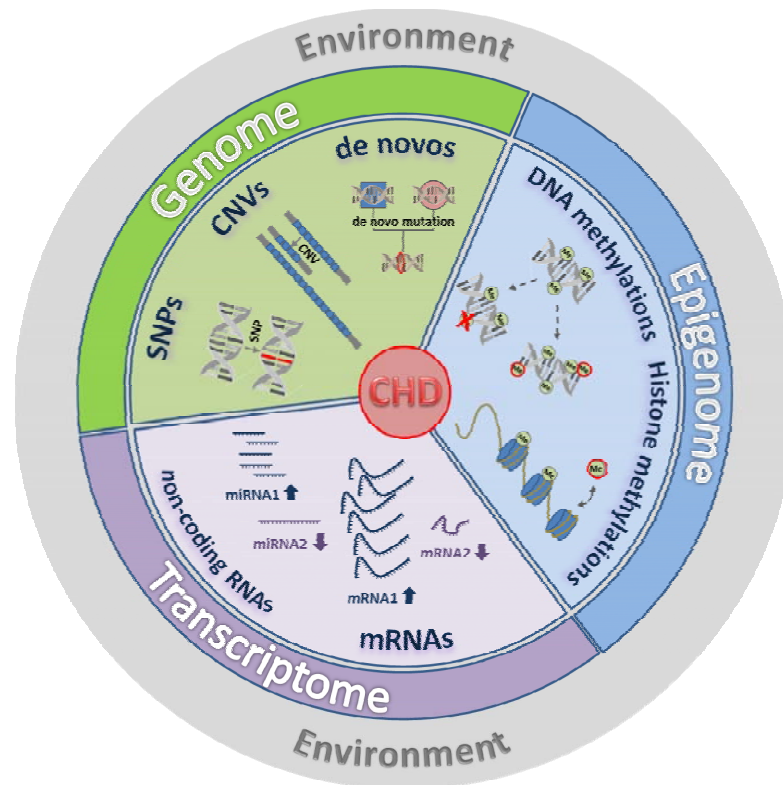
Fig. (2). Complex etiology of congenital heart defects. One must assume that a multitude of alterations happen which affect the genomic information directly (SNPs, CNVs, mutations, DNA methylation) or which alter the steady state of the transcriptome (dysregulation of coding and non-coding RNA species). Altogether these multiple factors generate a comprehensive genetic imbalance which ultimately results in the development of a distinct CHD phenotype.

with recurrent *de novo* mutations in both syndromic and non-syndromic patients. Finally, it is important to evaluate the functional relevance of these genetic findings in appropriate cellular test systems. The use of induced pluripotent stem cells derived from patients and unaffected first-degree relatives and the differentiation of such cells into the cardiac lineage would represent an ideal model to elucidate apparent functional anomalies *in vitro* respecting the complete patient-specific background.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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