

# VEGF Polymorphisms Are Associated With Endocardial Cushion Defects: A Family-Based Case-Control Study

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**ABSTRACT:** Endocardial cushion defects (ECDs) of the cardiac outflow tract are among the most common congenital heart disease phenotypes. VEGF is essential for endocardial cushion formation and derangements in VEGF synthesis lead to ECD. Three functional single nucleotide polymorphisms (SNPs) in the *VEGF* gene  $-2578 C>A$ ,  $-1154 G>A$ , and  $-634 G>C$  play a role in cardiogenesis. In a Dutch case-control family study of triads, 190 case and 317 control children with both parents, we investigated linkage and association between these *VEGF* SNPs and ECD. Allele frequencies for the three *VEGF* SNPs were comparable between ECD children and controls. However, *VEGF* alleles  $-2578 C$  and  $-1154 G$  were transmitted more frequently to children with ECD ( $p = 0.003$  and  $p = 0.002$ ), in particular perimembranous ventricular septal defects ( $p = 0.012$  and  $p = 0.006$ ). The  $-2578A/-1154A/-634G$  haplotype was associated with a reduced risk of ECD (OR 0.7; 95% CI, 0.6–1.0) and was significantly less transmitted to children with ECD ( $p = 0.002$ ). In a Dutch population, we show that the *VEGF* 2578 C,  $-1154 G$  alleles, and the AAG haplotype are associated with ECD. Possible *VEGF* gene-environment interactions exposures are discussed. (*Pediatr Res* 67: 23–28, 2010)

Congenital heart diseases are the most frequent major congenital malformations with a worldwide birth prevalence rate of over one million per year (1). Because of the high infant mortality and morbidity rates, the causes of these malformations should be identified and targeted for preventive strategies in the future (2). Most congenital heart diseases have a complex origin, in which interactions between subtle genetic variations and periconception exposures play a role (3). Vasculogenesis and heart development are dependent on the genetic constitution of the embryo derived from both parents, and the maternal genetically controlled nutritional, endocrine and metabolic environment (4). During cardiac development, the outflow tract is divided into the ascending aorta and the pulmonary trunk and aligned to the left and right ventricle, respectively. The formation of the endocardial cushions in the atrioventricular canal and outflow tract takes place by epithe-

lial-mesenchymal transformation, which is a key process in the development of the heart septa and valves. Numerous lines of research have implicated a strict spatiotemporal expression pattern of the *VEGF* gene in the control of endocardial cushion development (5). In addition to its tight regulation during cardiogenesis, *VEGF* gene expression is also regulated by environmental exposures. A nice example is that hypoxia induces *VEGF* gene expression and seems to contribute as such to the generation of major malformations in the atrioventricular-canal and outflow tract in mice (6). Hyperglycemia inhibits epithelial-mesenchymal transformation by reducing *VEGF* gene expression (7). Furthermore, hyperhomocysteinemia, which increases the risk of human congenital heart disease, was shown to up-regulate *VEGF* mRNA in a human endothelial cell line (8). Interestingly, *vegfl20/120* mutant mouse embryos, showing overexpression of the *VEGF*120-isoform and lacking the other *VEGF*-isoforms, develop endocardial cushion defects (ECDs), such as ventricular septal defects, pulmonary valve stenosis (PS), and Tetralogy of Fallot (TOF) (9,10).

In human, the *VEGF* gene is located on chromosome 6p21.3 and consists of eight exons that generate different isoforms through alternative splicing. *VEGF* gene expression shows a marked individual variability, which is partly determined by single nucleotide polymorphisms (SNPs). The most interesting functional SNPs that differentially express *VEGF in vitro* are *VEGF*  $-2578 C>A$  and  $-1154 G>A$ , located in the promoter (11), and *VEGF*  $-634 G>C$ , located in the 5' untranslated region of the gene (12). Initially, studies suggest associations between the *VEGF* SNPs  $-2578 C>A$ ,  $-1154 G>A$ , and  $-634 G>C$  and several congenital heart disease phenotypes (13–15).

In a large Dutch case-control family study of triads, *i.e.* child, mother, father, we investigated whether three functional SNPs in the *VEGF* gene, *i.e.*  $-2578 C>A$ ,  $-1154 G>A$ , and  $-634 G>C$ , affect the risk of ECD, being the most common subgroup of congenital heart disease phenotypes.

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**Abbreviations:** ECD, endocardial cushion defect; FBAT, family-based association test; HBAT, haplotype-based association test; PS, pulmonary valve stenosis; pVSD, perimembranous ventricular septal defect; SNP, single nucleotide polymorphism; TOF, Tetralogy of Fallot

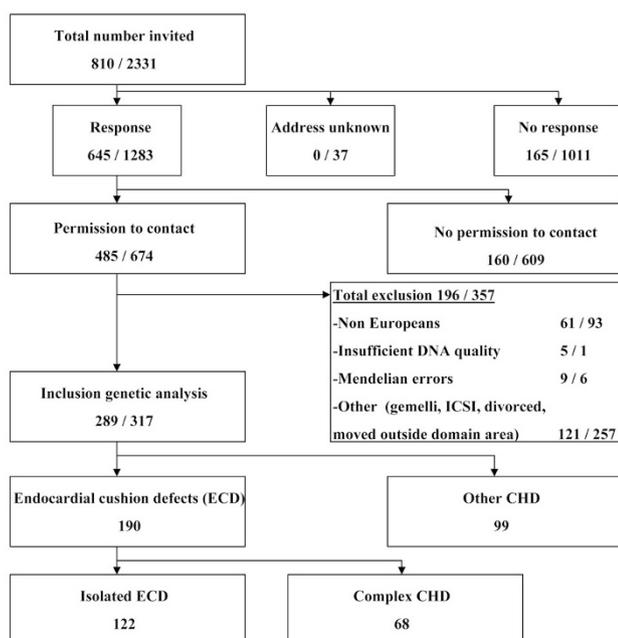
## MATERIALS AND METHODS

**Study design and population.** This study was embedded in the Dutch Haven study, which is an ongoing case-control family study of triads in the western part of the Netherlands, initiated to investigate the etiology and future prevention of congenital heart disease in which genetic and environmental factors are implicated. Details of the study design and population were described previously (16). Briefly, the domain population comprised case and control children, between 12 and 18 mo of age, living in the western part of the Netherlands. Children with a congenital heart disease were diagnosed by two pediatric cardiologists from the Erasmus MC, University Medical Center in Rotterdam, Leiden University Medical Center in Leiden, VU University Medical Center, and Amsterdam Medical Center in Amsterdam, in the Netherlands. In collaboration with the pediatric cardiologists, the case children with their parents were enrolled. In the Haven study, we included case families of which the index child had a congenital heart defect in which gene-environment interactions are implicated in the etiology (17–19). VEGF is reported to play a role in endocardial cushion formation. Therefore, in the current analysis of associations between SNPs in the *VEGF* gene and congenital heart defects we selected from the total case group of the Haven study, only the ECD phenotypes, *i.e.* perimembranous ventricular septal defect (pVSD), PS, TOF, aortic valve stenosis (AoS), and atrioventricular septal defect (AVSD). Figure 1 depicts the flow chart of the study population. Between October 2003 and January 2007, we ascertained 289 children with congenital heart disease and 317 control children with their parents. In the current analysis, only Dutch native children and children from other European origin were included to increase ethnic homogeneity. Children were classified as Dutch natives when both parents and grandparents are born in the Netherlands or one of the parents is born in another country, but both grandparents are born in the Netherlands. If one of the parents or grandparents is born in a European country or is from European origin and living in the United States, Australia, or Indonesia, we classified the child in the category European Others (20). Non-European children and families with Mendelian inconsistencies or unsuccessful genotyping for all three SNPs were also excluded. In total, SNP data from 190 case-children with ECD and both parents and 317 control-children with both parents were available for the analyses. All phenotypes were ascertained by ultrasound and/or cardiac catheterization and/or surgery and diagnosed according to the International Pediatric and Congenital Cardiac Code (21). The ECD group consisted of 122 children with isolated and 68 with complex ECD. Complex ECD was defined as ECD with the co-occurrence of any other structural congenital anomaly. The group of complex ECD was further subdivided into 46 children of which the ECD was part of a syndrome: Trisomy 21 ( $n = 34$ ), deletion 22q13 ( $n = 1$ ), deletion 22q11 ( $n = 2$ ), insertion 1 > 3 ( $n = 1$ ), Noonan syndrome ( $n = 1$ ), CHARGE syndrome ( $n = 1$ ), Duane syndrome ( $n = 1$ ), Turner syndrome ( $n = 2$ ),

VACTRL ( $n = 1$ ), Alagille syndrome ( $n = 1$ ) and Beckwith Wiedemann syndrome ( $n = 1$ ), and 22 children with ECD and other anomalies that could not be traced to a syndrome, such as hydronephrosis, hip dysplasia, hypospadias, cleft lip and/or palate, pyloric stenosis, clubfoot, and deafness. Control children and their parents were enrolled in collaboration with public child health centers in the Rotterdam area. The Dutch health care system includes a standardized and regular check up of all newborns for health, growth, and development by physicians trained in child health care. Children were eligible as controls if they did not have a major congenital malformation or chromosomal defect according to the medical records from the regular check up at the child health center. Both cases and controls were invited to participate at a fixed study moment at 16 mo of age of the child. At that time, the diagnosis of congenital heart diseases is confirmed. All children originated from the western part of the Netherlands. None of the families was related, all were familiar with the Dutch language in speaking and reading, and none of the children has been adopted. Potential participating parents were informed about the genetic research questions of the Haven study. Only children of whom the parents confirmed to be both the biologic parent participated. All mothers completed a general questionnaire from which we extracted for the current analyses demographic data, child's gender, family history of congenital heart disease, and ethnicity. The study protocol was approved by the Central Committee of Research in Humans in The Hague, the Netherlands, 2003, and the local Medical Ethical Committees of the four participating hospitals, 2003. Written informed consent was obtained from all parents, on behalf of their child as legal representatives.

**Genotyping.** DNA was isolated from EDTA blood with a Total Nucleic Acid Extraction kit on a MagNAPure LC (Roche Molecular Biochemicals, Mannheim, Germany). DNA was isolated from buccal swabs from four case-children, one case-father, and one control-father, due to logistical problems or failures in blood sampling. Genotypes were determined in 1–2 ng of genomic DNA with TaqMan allelic discrimination assays and analyzed using the software SDS 2.2.2 (Applied Biosystems, Foster City, CA). Case and control triads were genotyped for *VEGF* SNPs at positions  $-2578 C>A$ ,  $-1154 G>A$ , and  $-634 G>C$ , numbered from the translation start site. Primers and probes of  $-2578 C>A$  (rs699947),  $-1154 G>A$  (rs1570360), and  $-634 G>C$  (rs2010963) were obtained from Applied Biosystems (<http://www.appliedbiosystems.com>). Each genotype plate contained no DNA template (water) controls. To check consistency, we randomly chose 5% of samples and repeated the measurements. Reproducibility was 100%.

**Statistical analysis.** General characteristics were compared between children with ECD and controls. The comparisons between dichotomous variables, presented as numbers and percentages, were tested by  $\chi^2$  tests. Frequencies of *VEGF* alleles and genotypes were calculated for children with ECD and controls, and haplotype frequencies were estimated using UNPHASED 3.0 (22).  $D'$  and  $r^2$  values of linkage disequilibrium (LD) between SNPs were estimated using Haploview 3.3.2 (23). Deviations from Hardy-Weinberg equilibrium (HWE) were tested using  $\chi^2$  tests with one degree of freedom.  $\chi^2$  tests were also used to evaluate differences in the distributions of alleles, genotypes, and haplotypes between case and control children and separately for each ECD phenotype. A



**Figure 1.** Study population flowchart. Numbers are the absolute numbers of case mothers/control mothers. CDH, congenital heart disease.

**Table 1.** General characteristics of the case-children with ECD and control-children

	ECD	Controls
Child	$n = 190$	$n = 317$
Age at the study moment in months, median (interquartile range)	16.2 (15.1–19.2)	16.1 (15.1–18.0)
Boys, $n$ (%)	96 (51)	170 (54)
Family history for CHD*, $n$ (%)	18 (10)	17 (5)
Ethnicity†, $n$ (%)		
Dutch natives	177 (93)	289 (91)
European Others	13 (7)	28 (9)

\* Any congenital heart disease of family members in the first, second, or third degree.

† Children were classified as Dutch natives when both parents and grandparents are born in the Netherlands or one of the parents is born in another country, but both grandparents are born in the Netherlands. If one of the parents or grandparents is born in a European country, or is from European origin and living in the United States, Australia, or Indonesia, we classified the child in the category European Others.

CHD, congenital heart disease.

**Table 2.** Distribution of the VEGF genotypes in case-children with ECD and control-children

VEGF SNPs	ECD n = 185	Controls n = 312	$\chi^2$	Fisher's p	OR (95% CI)
-2578					
AA	44 (24)	88 (28)	2.52	0.28	0.8 (0.6–1.1)
CA	88 (48)	153 (49)			0.8 (0.5–1.2)
CC	53 (29)	71 (23)			1.0 (reference)
HWE	—	0.77			
p value					
C-allele	194 (52) n = 187	295 (47) n = 307	2.38	0.12	
-1154					
AA	18 (10)	43 (14)	2.32	0.31	0.8 (0.6–1.1)
GA	79 (42)	130 (42)			0.9 (0.6–1.3)
GG	90 (48)	134 (44)			1.0 (reference)
HWE	—	0.20			
p value					
G-allele	259 (69) n = 184	398 (65) n = 303	1.55	0.21	
-634					
CC	22 (12)	39 (13)	0.26	0.88	1.0 (0.7–1.3)
GC	85 (46)	133 (44)			1.1 (0.7–1.6)
GG	77 (42)	131 (43)			1.0 (reference)
HWE	—	0.57			
p value					
G-allele	239 (65)	395 (65)	0.00	1.00	

Values are expressed as n (%).  
ECD, outflow tract defect.

logistic regression model was used to estimate the risk of the pooled group of ECD phenotypes. OR and 95% CI were calculated. Statistical analysis was performed using SPSS software (version 12.0 SPSS Inc., Chicago, IL). We applied the family-based association test (FBAT), a transmission disequilibrium test (TDT)-like test statistic, to investigate linkage and association for VEGF alleles (FBAT) and haplotypes (haplotype-based association test [HBAT]) (<http://www.biostat.harvard.edu/~fbat/fbat.htm>) (24). FBAT analyses were performed under an additive model. The 1-POET test, which is an extension of the TDT statistic, was used to detect a possible parent-of-origin effect (25). All tests were two-tailed and p values were Bonferroni adjusted to account for multiple tests.

## RESULTS

The general characteristics of children with ECD and controls are presented in Table 1. The distributions of age, gender, ethnicity, and family history of congenital heart disease were comparable between the children with ECD and controls.

Moderate linkage disequilibrium was shown between VEGF -2578 C>A and -1154 G>A ( $r^2 = 0.52$ ); between VEGF -2578 C>A and -634 G>C ( $r^2 = 0.49$ ); and between VEGF -1154 G>A and -634 G>C ( $r^2 = 0.26$ ). The distributions of the VEGF alleles and genotypes are presented in Table 2. None of the genotype frequencies in the control population deviated from HWE. The genotype distributions of VEGF -2578 C>A, -1154 G>A, and -634 G>C were not significantly different between the children with ECD and controls. The VEGF -2578 C and -1154 G alleles were more frequently present, albeit not signifi-

**Table 3.** Distribution of the VEGF genotypes in case-children with ECD, stratified for ECD phenotypes, and control-children

VEGF SNPs	ECD					Controls n = 312
	pVSD n = 79	PS n = 42	TOF n = 29	AVSD n = 29	AoS n = 6	
-2578						
AA	15 (19)	12 (29)	6 (21)	9 (31)	2 (33)	88 (28)
CA	44 (56)	14 (33)	16 (55)	11 (38)	3 (50)	153 (49)
CC	20 (25)	16 (38)	7 (24)	9 (31)	1 (17)	71 (23)
$\chi^2$ ; p value*	0.25	0.07	0.68	0.47	0.93	
HWE p value	—	—	—	—	—	0.77
C-allele	84 (53)	46 (55)	30 (52)	29 (50)	5 (42)	295 (47)
$\chi^2$ ; p value†	0.16	0.18	0.49	0.65	0.72	
	n = 79	n = 43	n = 29	n = 30	n = 6	n = 307
-1154						
AA	6 (8)	6 (14)	2 (7)	3 (10)	1 (17)	43 (14)
GA	36 (46)	13 (30)	11 (38)	15 (50)	4 (67)	130 (42)
GG	37 (47)	24 (56)	16 (55)	12 (40)	1 (17)	134 (44)
$\chi^2$ ; p value*	0.31	0.27	0.39	0.68	0.40	
HWE p value	—	—	—	—	—	0.20
G-allele	110 (70)	61 (71)	43 (74)	39 (65)	6 (50)	398 (65)
$\chi^2$ ; p value†	0.24	0.28	0.16	1.00	0.36	
	n = 79	n = 43	n = 29	n = 27	n = 6	n = 303
-634						
CC	9 (11)	6 (14)	2 (7)	4 (15)	1 (17)	39 (13)
GC	43 (54)	16 (37)	14 (48)	10 (37)	2 (33)	133 (44)
GG	27 (34)	21 (49)	13 (45)	13 (48)	3 (50)	131 (43)
$\chi^2$ ; p value*	0.24	0.71	0.64	0.79	0.87	
HWE p value	—	—	—	—	—	0.57
G-allele	97 (61)	58 (67)	40 (69)	36 (67)	8 (67)	395 (65)
$\chi^2$ ; p value†	0.35	0.75	0.54	0.81	1.00	

Values are expressed as n (%), tested between case-children with ECD and controls by  $\chi^2$ .

\* Comparison of genotypes between cases and controls.

† Comparison of alleles between cases and controls.

ECD, outflow tract defect.

**Table 4.** Distribution of VEGF haplotypes in case-children with ECD and control-children

VEGF haplotypes -2578/-1154/-634	ECD	Controls	Fisher's		OR (95% CI)
			$\chi^2$	<i>p</i>	
AAG	114.4 (30)	225.1 (36)	3.92	0.05	0.76 (0.58–1.00)
CGC	133.4 (35)	220.2 (35)	0	1	—
CGG	65.0 (17)	82.4 (13)	3.07	0.08	1.37 (0.96–1.95)
AGG	65.1 (17)	104.8 (17)	0	1	—

Values are expressed as *n* (%).

ECD, outflow tract defects.

**Table 5.** Family-based association analysis of VEGF alleles transmitted from heterozygous parents to case-children with the separate ECD phenotype

	Allele VEGF	Frequency	FBAT		FBAT-o	
			Informative trios	<i>P</i>	Informative trios	<i>p</i>
ECD	-2578 C	0.490	136	0.015	366	0.003*
	-1154 G	0.663	131	0.017	331	0.002*
	-634 G	0.648	132	0.125	345	0.066
pVSD	-2578 C	0.490	64	0.052	294	0.012
	-1154 G	0.664	59	0.039	259	0.006
	-634 G	0.639	63	0.185	276	0.099
PS	-2578 C	0.494	32	0.170	262	0.060
	-1154 G	0.668	32	0.217	232	0.063
	-634 G	0.647	28	0.398	241	0.256
TOF	-2578 C	0.489	18	0.239	248	0.109
	-1154 G	0.670	19	0.221	219	0.084
	-634 G	0.646	19	0.127	232	0.081
AVSD	-2578 C	0.490	19	0.564	249	0.300
	-1154 G	0.663	18	0.835	218	0.456
	-634 G	0.642	19	0.852	232	0.892
AoS	NT					

FBAT-o incorporates information from control trios.

\* *p* values surviving Bonferroni correction for  $5 \times 3$  independent tests, with  $\alpha = 0.0033$ .

NT, sample size is too low; ECD, outflow tract defect.

cantly, in children with ECD than in controls. The distribution of the genotypes stratified per ECD phenotype is summarized in Table 3. In children with PS, the distributions of the -2578 C>A variants deviated from HWE, and in these children, the C-allele tended to be more frequently present than in controls ( $p = 0.07$ ). We inferred haplotypes from the three VEGF polymorphisms and compared the frequencies. Four of the eight possible haplotypes occurred at an appreciable frequency in both children with ECD and controls, *i.e.* AAG, CGC, CGG, and AGG whereby each letter refers to the allele of the -2578, -1154, and -634 SNP, respectively (Table 4). Consistent with the genotype distributions, the haplotype analyses revealed that the CGG haplotype was present in 17% of children with ECD and in 13% of control children ( $p = 0.08$ ). The AAG haplotype was associated with a reduced risk of the ECD phenotype (OR, 0.8; 95% CI, 0.6–1.0).

The results of the FBAT analyses are depicted in Table 5 and point out that the -2578C and -1154G alleles were transmitted to children with an ECD more frequently than expected by Mendelian inheritance (59% of all transmissions). FBAT-o, which also incorporates information from the control triads, revealed even stronger evidence for over transmission of the -2578 C allele ( $p = 0.003$ ) and the -1154 G allele ( $p = 0.002$ ) to offspring with the ECD phenotype. These *p* values survived the rather conservative Bonferroni correction for multiple testing of  $5 \times 3$  independent test with  $\alpha = 0.0033$ . Stratification for five separate ECD phenotypes revealed that the -2578 C allele ( $p = 0.012$ ) and the -1154 G allele ( $p = 0.006$ ) were significantly overtransmitted to children with pVSD. Consistently, the HBAT-o results suggested that the CGC haplotype was overtransmitted to children with ECD ( $p = 0.057$ ), and the AAG haplotype was significantly

**Table 6.** Family-based association analysis of VEGF haplotypes transmitted from parents to case-children with the separate ECD phenotype

	VEGF haplotypes -2578/-1154/-634	Frequency	HBAT		HBAT-o		HBAT - <i>p</i> <i>p</i>
			Informative trios	<i>p</i>	Informative trios	<i>p</i>	
ECD	CGC	0.344	113.9	0.063	294.8	.057	0.064
	AAG	0.330	109.9	0.008	288.8	0.002*	0.007
	AGG	0.173	76.0	0.612	202.9	0.492	0.611
	CGG	0.148	78.0	0.538	184.0	0.299	0.489
pVSD	CGC	0.354	56	0.056	239	0.045	0.060
	AAG	0.327	55	0.028	231	0.006	0.022
	AGG	0.172	41	0.764	169	0.629	0.699
	CGG	0.142	35	0.873	144	0.573	0.828
PS	CGC	0.352	20	0.117	191	0.090	0.098
	AAG	0.322	26	0.366	192	0.129	0.400
	AGG	0.174	15	0.819	142	0.946	0.743
	CGG	0.151	20	0.853	129	0.840	0.741
TOF	CGC	0.350	18	0.276	189	0.220	0.371
	AAG	0.320	15	0.221	181	0.090	0.322
	AGG	0.180	13	0.816	140	0.944	0.717
	CGG	0.149	14	0.590	123	0.435	0.462
AVSD	CGC	0.359	13	0.513	184	0.650	0.614
	AAG	0.326	12	0.405	178	0.227	0.449
	AGG	0.174	10	0.808	137	0.709	0.654
	CGG	0.140	11	0.166	120	0.113	0.205
AoS	NT	—	—	—	—	—	—

HBAT-o incorporates information from control trios.

*p* value surviving Bonferroni correction for  $5 \times 4$  independent tests, with  $\alpha = 0.0025$ .

HBAT-p, 10,000 permutations; NT, sample size is too low; ECD, outflow tract defect.

less frequently transmitted than expected to children with ECD (41% of transmissions, which was significant at an Bonferroni  $\alpha$ -level of 0.0025) (Table 6), in particular to children with pVSD ( $p = 0.022$ ). Empirical  $p$  values obtained by permutation analyses (HBAT-p) substantiated these associations. The global  $p$  value with four degrees of freedom for differences in the transmission of the four haplotypes to children with ECD was  $p = 0.03$ . We did not find any evidence for a parent-of-origin effect of risk alleles in the *VEGF* candidate loci (data not shown).

## DISCUSSION

This study provides evidence for an association between the *VEGF* gene, in particular, the *VEGF*  $-2578$  C and  $-1154$  G alleles and AAG haplotype, and ECD in human.

Recently, Vannay *et al.* (14) demonstrated a higher frequency of the *VEGF*  $-634$  C allele in Hungarian children with congenital heart disease. They included several congenital heart disease phenotypes with valvular and/or septal defects, which may explain the different results. In a Chinese study, the *VEGF*  $-634$  C allele was shown to reduce the risk of a child with the pVSD phenotype (15). Our sample size of 79 children with pVSD and 317 controls was too limited to detect the association with this phenotype (power of 41%, OR 0.7, risk allele frequency 35%, type I error of 0.05, congenital heart disease population risk of 0.008). Lambrechts *et al.* (13) reported an increased risk of the *VEGF*  $-2578/-1154/-634$  AAG haplotype for both nonsyndromic and DiGeorge syndrome-related TOF. However, we show that the same AAG haplotype was associated with a reduced risk for in particular ECD. In our study, the *VEGF*  $-2578$  A and  $-1154$  A alleles were less frequent in children with TOF, although not significantly different from controls. The genetic differences that may exist between our Dutch population and their mixed population of Caucasians, Afro-Americans, Hispanics, and subjects from unknown origin might explain the differences in results. Furthermore, it cannot be excluded that selective survival is confounding their results as no information was provided on the age of the children with congenital heart disease at the moment of investigation. Moreover, maternal exposures in early pregnancy that seem to influence *VEGF* expression might also have led to the different results.

The effect of the studied *VEGF* SNPs on the expression in human, particularly the *VEGF* isoforms, is not fully understood. In *vegfl20/120* mouse embryos, lacking the 164 and 188 isoforms, a spatiotemporal increase of VEGF coincides with hyperplasia of the outflow tract cushions and abnormally high levels of apoptosis. This manifests in later stages than TOF, pulmonary stenosis, and ventricular septal defects (10). In humans, higher VEGF production was observed in cells from individuals with the  $-2578$  C and  $-1154$  G alleles compared with  $-2578$  A and  $-1154$  A individuals in stimulated peripheral blood mononuclear cells from healthy volunteers (26). Individuals homozygous for the haplotypes containing the  $-1154$  AA and  $-2578$  AA genotypes also showed lower circulating VEGF than heterozygous individuals, whereas the *VEGF* AAG haplotype resulted in lower plasma

*VEGF* concentrations (27). We speculate that the AAG haplotype is associated with a reduced risk for ECD by modifying the spatiotemporal *VEGF* expression during heart development.

Our findings suggest that the polymorphic promoter region of the *VEGF* gene, in particular the  $-2578$  C and  $-1154$  G alleles, contribute to a genetic predisposition to ECD. The genetic variants might cause a spatiotemporal increase in VEGF expression during endocardial cushion formation, superimposed on the developmental regulated program of expression. Hypoxia, hyperglycaemia, and hyperhomocysteinemia are suggested to modify VEGF expression during cardiogenesis (5,6,8). The interaction between *VEGF* and homocysteine is of particular interest because both epidemiologic and experimental studies showed that maternal hyperhomocysteinemia increases the risk of offspring with in particular ECD (16–18). Folate shortage results in hyperhomocysteinemia and both folic acid and homocysteine affect the behavior of neural crest cells that are implicated in endocardial cushion formation (28). Periconception use of folic acid supplements was shown to reduce the risk of in particular neural crest-related congenital heart disease (19). VEGF functions as an endothelial-cell specific growth factor and affects epithelial, mesenchymal, and neural cells. It is unknown whether VEGF also influences neural crest cells. However, in future studies with large sample sizes these interactions should be further studied.

Some limitations and strengths of the study have to be addressed. Our dataset consisted of a relatively large set of ECD; however, sample sizes of the individual ECD phenotypes were relatively small, especially that of AoS. Therefore, with an *a priori* hypothesis and to minimize type I errors possibly occurring when testing the individual phenotypes, at first we pooled the five ECD phenotypes as they share similarities in pathogenetic background. However, as there may be differential effects between the phenotypes, we also presented the FBAT and HBAT results per ECD phenotype. We acknowledge the selection of only surviving children of 16 mo of age with and without a congenital heart defect that may have resulted in an over- or underestimation of the risk estimates. Only a few data have been published on the mortality rates of congenital heart defects in the first year of life. To provide some estimation of the impact on the risk estimates, we calculated that the overall mortality rate of ECD in our hospital over the previous years was on average 2%. The European Association of Cardio-Thoracic Surgeons (EACTS) reported mortality rates varying between 0.76% for AoS and 6.98% for complete AVSDs (29). The overall infant mortality rate in the first year of life in the developed world is 0.8%. Therefore, it is not very likely that the selection of only surviving case and control children had a great impact on the conclusions of this study. The strength of our study is the detailed description of the ECD phenotypes and the ethnic homogeneity of case and control families. Moreover, the *VEGF* genotypes are reliable as the distributions in the controls are largely comparable with healthy control subjects in other European study populations (27). Bonferroni correction is rather conservative when considering SNPs in linkage disequilibrium and may increase the risk of false negative results. However, to minimize the risk for type I errors,

Bonferroni adjusted probability values were calculated for  $5 \times 3$  and  $5 \times 4$  independent tests for alleles and haplotypes, respectively. It is of note that the associations between the *VEGF* -2578 C ( $p = 0.003$ ) and -1154 G ( $p = 0.002$ ) allele as well as the AAG haplotype ( $p = 0.002$ ) with ECD, as revealed from the FBAT-o and HBAT-o analyses, were of such magnitude that they would remain significant even after Bonferroni correction. The FBAT looks for distortions in the transmission frequencies of a given allele, compared with the assumption of random transmission. A TDT-like design, such as FBAT, is an attractive method because it is robust against population admixture or stratification (30). Another advantage of FBAT over standard TDT is the FBAT-o option, which also incorporates information on the control triads.

The observed associations between the *VEGF* polymorphisms at position -2578 and -1154, the AAG haplotype, and ECD phenotypes require replication in an independent study group. The rapid expansion of knowledge regarding genetic profiling will in future lead to a more detailed characterization of congenital heart disease. Large studies addressing interactions between *VEGF* and periconception environmental exposures as well as those investigating the signaling pathway upstream and downstream of *VEGF* during cardiac cushion formation should be encouraged to modify ECD risk in future.

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