CONGENITAL HEART DISEASE (CHD) PRESENTS A HUGE MEDICAL PROBLEM, AS IT AFFECTS BETWEEN TWO AND EIGHT NEWBORN CHILDREN PER 100 LIVE BIRTHS. RISK FACTORS INCLUDE ALCOHOL AND DRUG CONSUMPTION AS WELL AS GENETIC DEFECTS. HOWEVER, CHROMOSOMAL AND SINGLE GENE DEFECTS CAUSE ONLY A RELATIVELY MINOR PROPORTION OF CASES AND, THUS, MOST CHD IS CONSIDERED TO BE MULTI-FACTORY IN ORIGIN, WITH VARIOUS GENES INTERACTING WITH EACH OTHER OR WITH ENVIRONMENTAL FACTORS TO DETERMINE DISEASE LIABILITY. TO DATE, NONE OF THE CHD GENETIC SUSCEPTIBILITY FACTORS HAVE BEEN DISCOVERED.

TETRALOGY OF FALLOT (TOF) IS A COMMON FORM OF CHD, CHARACTERISED BY A SUBAORTIC VENTRICULAR SEPTUM DEFECT (VSD), AN OVERRIDING AORTA, A RIGHT VENTRICULAR OUTFLOW TRACT OBSTRUCTION, AND RIGHT VENTRICULAR HYPERTROPHY. TOF OCCURS IN 4.21 OF EVERY 10 000 BIRTHS AND IS THE MOST COMMON TYPE OF CHD WITH CYANOSIS AFTER 1 YEAR OF LIFE. TOF MAY OCCUR AS PART OF THE DIDGEORGE SYNDROME (DGS) WHICH IS CAUSED BY DELETIONS OF CHROMOSOME 22q11 AND CHARACTERISED BY CONOTRUNCAL CARDIAC, CRANIOFACIAL, THYMIC, AND PARATHYROID ANOMALIES. HOWEVER, IN MOST CASES (IN 2.65 PER 10 000 CHILDREN) TOF OCCURS AS AN ISOLATED DEFECT. MUTATIONS IN THE NKX2.5 GENES HAVE BEEN FOUND IN ONLY A FEW PERCENTAGES OF CASES WITH ISOLATED, NON-SYNDROMIC TOF AND THUS, THE GENETIC ETIOLOGY IN THE LARGE MAJORITY OF THESE CASES REMAINS ENTIRELY UNKNOWN. BY USING A MULTI-GENETIC APPROACH, WE RECENTLY DISCOVERED THAT VEGF IS A MODIFIER OF DGS. WE THEREFORE ASSESSED HERE WHETHER VEGF MIGHT BE A MODIFIER OF THE CARDIAC BIRTH DEFECTS IN SUBJECTS WITH ISOLATED, NON-SYNDROMIC TOF.

METHODS
Study design and participants
To examine whether VEGF gene variations are associated with TOF, we used the transmission disequilibrium test (TDT) to analyse linkage disequilibrium of single nucleotide polymorphisms (SNPs) in the VEGF gene in trios of parents and their proband affected with TOF. A total of 148 probands (84 males, 64 females; 73 Caucasians, 58 Caucasians of Flemish origin, four Afro-Americans, two Hispanics, and 11 trios of unknown or mixed ethnic origin) and their parents were studied. Ninety trios were recruited by the Children’s Hospital in Philadelphia, USA, and 58 trios by the University Hospital in Leuven, Belgium. Criteria for diagnosis of TOF included the presence of a subaortic VSD, an overriding aorta, a right ventricular outflow tract obstruction (infundibular, valvar, supravalvar, or a combination), and right ventricular hypertrophy. Subjects with a 22q11 deletion were excluded from the analysis. To determine whether the association was specific for TOF, we also studied probands with transposition of the great arteries (TGA). A total of 40 probands (27 males and 13 females; 29 Caucasians, one Asian, one Hispanic, three Afro-Americans, and six of mixed or unknown ethnic origin) and their parents were recruited at the Children’s Hospital, Philadelphia. Thirty seven patients were diagnosed with a D-TGA, which is the more common form of TGA and consists of a complete inversion of the great vessels, so that the aorta arises from the right ventricle and the pulmonary artery from the left ventricle, while three patients were diagnosed with an L-TGA, in which the ventricles are inverted. The study was approved by the Institutional Review Board for the Protection of Human Subjects and the Research Ethics Board in Philadelphia and Leuven, respectively, and written informed consent to participate in the study was obtained from all subjects or their legal representatives.

SNP genotyping
Three SNPs in the VEGF gene regulatory regions were studied: the first two SNPs (−2578C/A and −1154G/A; positions relative to the VEGF translation start site) are located in the VEGF promoter and the third SNP (−634G/C) is located in the VEGF leader sequence. Characterisation of the VEGF genotypes was performed by TaqMan minor groove binding probes and real time allelic discrimination of VEGF SNPs. The following forward and reverse primers were used for the −1154G/A SNP: 5’-CCGCTACGACGGAGCTTTT-3’ and 5’-AGGCTTCGCTACTCTCCTCT-3’.

REFERENCES
patients with TOF more frequently than expected by mendelian inheritance, that is in 59% of all transmissions (table 1). Thus, alleles associated with low levels of VEGF were significantly overtransmitted towards patients with TOF. In European populations, the −2578, −1154, and −634 VEGF SNPs are in linkage disequilibrium and only four VEGF haplotypes are commonly observed, that is AAG, AGG, CGG, and CGC (each letter refers to the SNP of the −2578, −1154, and −634 allele, respectively; see table 2 for more explanation).4 Despite the genetic heterogeneity of the American population, the same four haplotypes (that is, AAG, AGG, CGG, and CGC) present in European populations also predominated in the American population.4 Haplotypes could therefore be converted into genotypes and the parent to offspring transmission of haplotypes unambiguously determined for all families. The AAG haplotype, which lowers VEGF expression,4 was transmitted in 61% of all transmissions (p = 0.008; table 2). The AGG and CGG haplotypes were not significantly overtransmitted to affected children, though there was a tendency for the AGG haplotype to be overtransmitted (table 2).

To further assess whether the association of VEGF with isolated, non-syndromic TOF was not false-positive, we performed a meta-analysis by calculating the pooled odds ratio for the association of the common AAG haplotype with isolated, non-syndromic forms of TOF (present study) and with TOF associated with DGS (replication study). By meta-analysis, the AAG haplotype was found to increase the risk 1.8-fold under a random effect model (CI = 1.3 to 2.5; p = 0.0008; test of homogeneity Cochran Q; p = 0.15). Thus, the low-VEGF AAG haplotype conferred an increased risk for TOF, both when occurring as an isolated, non-syndromic defect as well as in the context of DGS.

To determine whether this association was specific for TOF, we also studied probands with TGA and their parents. None of the VEGF alleles or haplotypes were transmitted significantly more often than expected by normal mendelian inheritance (table 3). This suggests that the association between the low-VEGF alleles and TOF was specific and that low-VEGF alleles do not simply predispose to CHD. A negligible role for VEGF in TGA may not be surprising, as this lesion does not belong to the typical spectrum of conotruncal defects in DGS patients and mouse models.14 In addition, there is little evidence for a genetic predisposition for TGA, as only very few familial cases have been reported to date12 and a recent study even failed to identify any recurrence among offspring of adults with TGA.2

### RESULTS

We previously established that the −2578A and −1154A alleles, which are located in the VEGF promoter, lower VEGF gene transcription, while the −634G allele, which is located in the VEGF 5′UTR, reduces internal ribosome entry site mediated VEGF expression and translation of the large VEGF isoform.4 Genotyping of the VEGF SNPs in trios of parents and their proband affected with TOF revealed that the −2578A, −1154A, and −634G alleles were transmitted to

<table>
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<tr>
<th>Gene variation</th>
<th>Risk allele</th>
<th>Allele frequency (%)</th>
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<th>χ²</th>
<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>−2578 C/A</td>
<td>−2578A</td>
<td>0.49</td>
<td>87 (0.59)</td>
<td>61 (0.41)</td>
<td>4.6</td>
<td>0.016</td>
</tr>
<tr>
<td>−1154 G/A</td>
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<td>0.32</td>
<td>73 (0.59)</td>
<td>50 (0.41)</td>
<td>4.3</td>
<td>0.019</td>
</tr>
<tr>
<td>−634 G/C</td>
<td>−634G</td>
<td>0.66</td>
<td>77 (0.59)</td>
<td>54 (0.41)</td>
<td>4.1</td>
<td>0.022</td>
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The exact number of transmitted and untransmitted risk alleles with their relative frequency between brackets is indicated.

5′-TGGGTAAATTTTCAGCTTGTAAC-3′; and for the −634G/C SNP: 5′-CAGAGACAAGTCTTGAAGAGAGA-3′ and 5′-CCCCAAAAGCAGGTCACTCA-3′. The following labelled probes were used for the −1154G/A SNP: 5′-CCTACAGCCCTTCC-VIC-3′ and 5′-CCTACAGCCCTCCTC-FAM-3′; and for the −634G/C SNP: 5′-CTTCGCTTGTCTGGCTGGCTGCFA-M-3′ and 5′-TGCCCTGTCCTTTGGCTGC-TET-3′. The −2578C/A SNP was genotyped by pyrosequencing as described previously.4

### Statistical analysis

The GeneHunter transmission disequilibrium command was used to calculate the non-parametric linkage statistic for the VEGF alleles and haplotypes.3 Because previously obtained biological data suggested which low-VEGF alleles or haplotypes might increase the risk for TOF, we knew in advance the direction of the test.34 We therefore calculated one sided p values by halving the two sided probabilities generated by GeneHunter for the AAG haplotype and for the VEGF alleles in the direction of their appearance in the AAG haplotype. For the meta-analysis, we compared the number of AAG haplotypes in cases and controls from the DGS study (replication study) and calculated the crude odds ratio and 95% confidence interval (CI) by χ² analysis with 1 degree of freedom.4 The odds ratio for the TDT study (present study) was calculated by treating the number of transmissions of the AAG haplotype as the number of occurrences of that haplotype in cases and considering the controls to be a very large population with equal numbers of the haplotype (to reflect the expected 50:50 transmission ratio).4 As both studies measure a different true effect (that is, the risk in people knowing in advance the direction of the test).34 We therefore calculated one sided p values by halving the two sided probabilities generated by GeneHunter for the AAG haplotype and for the VEGF alleles in the direction of their appearance in the AAG haplotype.

Table 1: Transmission of VEGF alleles from heterozygous parents to TOF affected children

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Table 2: Transmission of VEGF haplotypes from heterozygous parents to TOF affected children

<table>
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<tr>
<th>Haplotype</th>
<th>Haplotype frequency (%)</th>
<th>Transmitted haplotypes</th>
<th>Untransmitted haplotypes</th>
<th>χ²</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAG</td>
<td>0.33</td>
<td>72 (0.61)</td>
<td>46 (0.39)</td>
<td>5.8</td>
<td>0.008</td>
</tr>
<tr>
<td>AGG</td>
<td>0.17</td>
<td>44 (0.55)</td>
<td>36 (0.45)</td>
<td>0.8</td>
<td>0.39</td>
</tr>
<tr>
<td>CGC</td>
<td>0.33</td>
<td>52 (0.41)</td>
<td>74 (0.59)</td>
<td>3.2</td>
<td>0.04</td>
</tr>
<tr>
<td>CGG</td>
<td>0.17</td>
<td>43 (0.47)</td>
<td>49 (0.53)</td>
<td>0.4</td>
<td>0.53</td>
</tr>
</tbody>
</table>

The VEGF haplotypes and their frequencies are presented. The exact number of transmitted and untransmitted haplotypes with their relative frequency between brackets is indicated. AAG: −2578A/−1154A/−634G haplotype; AGG: −2578A/−1154G/−634G haplotype; CGC: −2578C/−1154G/−634G haplotype.
neural, epithelial, and mesenchymal cells. Whether VEGF, most endothelial cell-specific growth factor, but numerous remain to be elucidated. VEGF was once considered to be the downstream pathways of VEGF involved in this process is required for cardiac morphogenesis, even though the myocardium, which precisely mark the sites of atrioventricular pattern. At the onset of chamber septation, developing heart occurs in a highly specific spatio-temporal formation of the atrioventricular canal and the remodelling in particular, of TOF? In mice Vegf expression in the developing heart occurs in a highly specific spatio-temporal pattern. At the onset of chamber septation, Vegf is up regulated in two diametrically opposed regions of the myocardium, which precisely mark the sites of atrioventricular canal formation. Vegf expression hotspots are also present in the fourth and sixth pharyngeal arches, preceding remodelling of the corresponding arch arteries. Thus, VEGF is expressed at sites known to play a critical role in the formation of the atrioventricular canal and the remodelling of the large thoracic arteries. That VEGF is also functionally involved and that VEGF levels must be tightly regulated in cardiac morphogenesis is evidenced by various genetic mouse models. For instance, lack of even a single Vegf allele disregulates embryonic heart formation, while newborn mice lacking the most critical Vegf isoform of DGS-like cardiac birth defects—several of them due to TOF. All these findings thus indicate that precise control of VEGF expression is required for cardiac morphogenesis, even though the downstream pathways of VEGF involved in this process remain to be elucidated. VEGF was once considered to be the most endothelial cell-specific growth factor, but numerous studies now indicate that this growth factor also affects neural, epithelial, and mesenchymal cells. Whether VEGF thus affects neural crest cells, epithelial arch endoderm cells, or other cell types involved in cardiac morphogenesis is an outstanding but intriguing question. Whatever the mechanism, the role of VEGF must be specific, as this growth factor is not associated with the transposition of the great arteries.

In conclusion, the present human genetic evidence that the AAG haplotype of the VEGF gene increases the risk for DGS associated and non-syndromic TOF, together with our previous functional in vivo data in genetic mouse and zebrafish models and with the in vitro findings that the “at risk” AAG haplotype lowers Vegf expression, suggests that VEGF is indeed a modifier of TOF.

**ACKNOWLEDGEMENTS**

We thank the patients and their families who consented to this study. The authors thank A Claeyss and S Jansen for technical support.

**DISCUSSION**

Both environmental and genetic risk factors are generally considered to contribute to CHD. However, single gene defects, such as mutations in TBX5, TBX3, GATA4, and NKX2.5, explain only very few CHD cases (<1–4%) and, even then, their clinical spectrum is often quite variable. It has, therefore, often been hypothesised, but never proven, that susceptibility to and the expressivity of most cases of CHD is in fact caused by genetic variations and several interacting susceptibility factors. The most significant finding of this study is that VEGF may well be such a modifier gene for TOF. Indeed, a low-VEGF haplotype (AAG), commonly occurring in up to a third of European and American populations, increased the risk for the development of an isolated, non-syndromic form as well as a syndromic form of TOF. To the best of our knowledge, this is the first evidence for an association between a modifier gene and any type of CHD.

How does VEGF modify the clinical spectrum of CHD and, in particular, of TOF? In mice Vegf expression in the developing heart occurs in a highly specific spatio-temporal pattern. At the onset of chamber septation, Vegf is upregulated in two diametrically opposed regions of the myocardium, which precisely mark the sites of atrioventricular canal formation. Vegf expression hotspots are also present in the fourth and sixth pharyngeal arches, preceding remodelling of the corresponding arch arteries. Thus, VEGF is expressed at sites known to play a critical role in the formation of the atrioventricular canal and the remodelling of the large thoracic arteries. That VEGF is also functionally involved and that VEGF levels must be tightly regulated in cardiac morphogenesis is evidenced by various genetic mouse models. For instance, lack of even a single Vegf allele disregulates embryonic heart formation, while newborn mice lacking the most critical Vegf isoform of DGS-like cardiac birth defects—several of them due to TOF. All these findings thus indicate that precise control of VEGF expression is required for cardiac morphogenesis, even though the downstream pathways of VEGF involved in this process remain to be elucidated. VEGF was once considered to be the most endothelial cell-specific growth factor, but numerous studies now indicate that this growth factor also affects neural, epithelial, and mesenchymal cells. Whether VEGF thus affects neural crest cells, epithelial arch endoderm cells, or other cell types involved in cardiac morphogenesis is an outstanding but intriguing question. Whatever the mechanism, the role of VEGF must be specific, as this growth factor is not associated with the transposition of the great arteries.

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**REFERENCES**


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<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>-2578 C/A</td>
<td>-2578 A</td>
<td>0.48</td>
<td>16 (0.47)</td>
<td>0.73</td>
<td>0.1</td>
<td>0.73</td>
</tr>
<tr>
<td>-1154 G/A</td>
<td>-1154 A</td>
<td>0.34</td>
<td>16 (0.48)</td>
<td>0.03</td>
<td>0.86</td>
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<tr>
<td>-634 G/C</td>
<td>-634 G</td>
<td>0.73</td>
<td>16 (0.55)</td>
<td>0.3</td>
<td>0.58</td>
<td></td>
</tr>
</tbody>
</table>

The exact number of transmitted and untransmitted risk alleles with their relative frequency between brackets is indicated. Two sided p values are presented.
Low expression VEGF haplotype increases the risk for tetralogy of Fallot: a family based association study

D Lambrechts, K Devriendt, D A Driscoll, E Goldmuntz, M Gewillig, R Vlietinck, D Collen and P Carmeliet

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