Rare variants in \textit{KDR}, encoding VEGF Receptor 2, are associated with tetralogy of Fallot

Doris Škorić-Milosavljević\textsuperscript{1,38}, Najim Lahrouchi\textsuperscript{1,38}, Fernanda M. Bosada\textsuperscript{2,38}, Gregor Dombrowsky\textsuperscript{3}, Simon G. Williams\textsuperscript{5}, Robert Lesurf\textsuperscript{5}, Fleur V. Y. Tjong\textsuperscript{1}, Roddy Walsh\textsuperscript{1}, Isshane El Bouchikhi\textsuperscript{6}, Jeroen Breckpot\textsuperscript{7}, Enrique Audain\textsuperscript{8}, Aho Ilgum\textsuperscript{9}, Leander Beekman\textsuperscript{10}, Ilham Rabb\textsuperscript{9,11}, Alanna Strong\textsuperscript{10,11}, Maximilian Muenke\textsuperscript{12}, Solveig Heide\textsuperscript{13}, Alison M. Muir\textsuperscript{14}, Mariam Hababa\textsuperscript{1}, Laura Cross\textsuperscript{15}, Dihong Zhou\textsuperscript{16}, Tomi Pastinen\textsuperscript{16}, German Competence Network for Congenital Heart Defects*, Elaine Zackai\textsuperscript{10}, Samir Atmani\textsuperscript{17,18}, Ilham Ratbi\textsuperscript{8,9}, Alanna Strong\textsuperscript{10,11}, Maximilian Muenke\textsuperscript{12}, Solveig Heide\textsuperscript{13}, Alison M. Muir\textsuperscript{14}, Mariam Hababa\textsuperscript{1}, Laura Cross\textsuperscript{15}, Amy Roberts\textsuperscript{24}, Elisabeth M. Lodder\textsuperscript{25}, Bernard D. Keavney\textsuperscript{4,26}, Sally-Ann B. Clur\textsuperscript{22}, Seema Mital\textsuperscript{27,28}, Marc-Philip Hitz\textsuperscript{3,29,30}, Vincent M. Christoffels\textsuperscript{2}, Alex V. Postma\textsuperscript{2,25,38} and Connie R. Bezzina\textsuperscript{1,38}

**PURPOSE:** Rare genetic variants in \textit{KDR}, encoding the vascular endothelial growth factor receptor 2 (VEGFR2), have been reported in patients with tetralogy of Fallot (TOF). However, their role in disease causality and pathogenesis remains unclear.

**METHODS:** We conducted exome sequencing in a familial case of TOF and large-scale genetic studies, including burden testing, in >1,500 patients with TOF. We studied gene-targeted mice and conducted cell-based assays to explore the role of \textit{KDR} genetic variation in the etiology of TOF.

**RESULTS:** Exome sequencing in a family with two siblings affected by TOF revealed biallelic missense variants in \textit{KDR}. Studies in knock-in mice and in HEK 293T cells identified embryonic lethality for one variant when occurring in the homozygous state, and a significantly reduced VEGFR2 phosphorylation for both variants. Rare variant burden analysis conducted in a set of 1,569 patients of European descent with TOF identified a 46-fold enrichment of protein-truncating variants (PTVs) in TOF cases compared to controls ($P = 7 \times 10^{-11}$).

**CONCLUSION:** Rare \textit{KDR} variants, in particular PTVs, strongly associate with TOF, likely in the setting of different inheritance patterns. Supported by genetic and in vivo and in vitro functional analysis, we propose loss-of-function of VEGFR2 as one of the mechanisms involved in the pathogenesis of TOF.

**INTRODUCTION**

Tetralogy of Fallot (TOF) is the most common form of cyanotic congenital heart defect (CHD).\textsuperscript{1,2} Although TOF can present in combination with extracardiac defects, in the majority of cases it presents as an isolated defect.\textsuperscript{2} An increased risk of CHD among first-degree relatives and offspring of TOF patients\textsuperscript{3,4} provides evidence for a genetic contribution to the disease etiology. A microdeletion on chromosome 22q11.2 is the most common genetic abnormality identified in patients with TOF, accounting for~15% of cases.\textsuperscript{5} In addition, several other genes, mainly encoding cardiac transcription factors,\textsuperscript{6,7} have been implicated in TOF, although these account for a minority of patients, and the majority of cases remain genetically elusive. Over the last few years, dysregulated vascular endothelial growth factor (VEGF) signaling h-
as been implicated in the pathogenesis of TOF. Exome sequencing studies have provided robust evidence that dominant, mainly truncating, pathogenic variants in FLT4, encoding VEGF receptor 3 (VEGFR3), are an important genetic cause of TOF.3,5,6 Furthermore, a causal role of rare variants in this gene has not been definitively established for CHD.

We conducted exome sequencing in a family with two children identified by whole-exome sequencing. We subsequently conducted large-scale genetic study in patients with TOF, including burden testing, and studies in gene-targeted mice and cell-based assays, to further explore the prevalence and nature of KDR genetic variation in patients with TOF and possible mechanisms leading to the etiology of TOF.

**MATERIALS AND METHODS**

A detailed description of the methods is available in the Online Supplemental Methods.

Genetic analysis and knock-in mouse model of index family with tetralogy of Fallot

We performed exome sequencing on DNA from two siblings of Moroccan descent affected by TOF with suspected recessive inheritance. Mouse lines harboring variants orthologous to the two KDR variants identified in the index family were generated by CRISPR/Cas9 targeting at Cyagen (Santa Clara, CA 95050-2709, USA).

**VEGFR2 phosphorylation assay**

VEGFR2 phosphorylation status was assessed by western blot in yolk sac cells from knock-in mice and transfected HEK 293T cells. Values were compared to wild-type values by one-sided t-test per condition. A p value < 0.05 was considered significant.

Additional patients with rare KDR variants

Patients with TOF were selected based on one of the following criteria, either (1) complex TOF, defined as TOF with absent pulmonary valve syndrome, pulmonary atresia, double outlet right ventricle, or with aortic arch abnormalities; or (2) TOF with a positive family history of CHD or (3) TOF patients of Moroccan descent. Patients from categories 1 and 2 were derived from the Dutch national biobank of adult patients with CHD.
(CONCOR) or recruited at the Amsterdam UMC (Netherlands) and at the Boston Children’s Hospital (USA). Patients from category 3 were recruited at the University Hospital of Fez (Morocco). KDR coding and copy-number variants were screened by Sanger sequencing and quantitative polymerase chain reaction (qPCR) respectively. In addition, we submitted KDR to the GeneMatcher database and considered both patients with CHD as well as patients with other phenotypes.

Rare variant association analysis

We compared the burden of rare KDR variants in patients of European descent with (1) unselected TOF (primary analysis) or (2) patients with any type of CHD other than TOF (secondary analysis); these comprised a broad spectrum of CHDs, varying from mild to severe defects, described in detail elsewhere and controls. Cases were drawn from four different CHD cohorts for which exome or genome sequence data was previously generated. The non-Finnish European (NFE) subset of gnomAD v2 was used as control data set.

We assessed rare variants in the canonical KDR transcript (ENST00000263923) in two different categories: protein-truncating variants (PTVs, i.e., nonsense, frameshift, and splice site variants) and missense variants. In addition, we analyzed the missense variants separately in different domains of VEGFR2 (Supplementary table 1). Burden comparisons were performed using Fisher’s exact test. We applied a stringent Bonferroni corrected p value of < 0.007 (i.e., 0.05/7 tests) as significance threshold.

RESULTS

Familial tetralogy of Fallot with biallelic KDR variants

The index family is a nonconsanguineous family of Moroccan descent with two children affected by a severe and complex form of TOF (Fig. 1a). Patient II-1 was diagnosed with a severe type of TOF, consisting of absent pulmonary valve syndrome and double outlet right ventricle (Fig. 1b). Patient II-3 was diagnosed with TOF with a severely hypoplastic pulmonary valve and a double outlet right ventricle, and a right-sided aortic arch. Detailed phenotypes are provided in Supplementary table 2.

There was no evidence of facial dysmorphisms, extracardiac manifestations, or neurodevelopmental delay in either child (current ages 17 and 7 years, respectively). Both parents (I-1 and I-2) and the unaffected sister (II-2) had normal echocardiograms and were healthy.

Exome sequencing, performed in II-1 and II-3, revealed 85 rare (minor allele frequency [MAF] < 0.1%) nonsynonymous or splice site variants that were shared among the two affected patients, none of which were homozygous. We excluded the presence of rare variants in a curated set of TOF genes (n = 77; Supplementary table 3). Because of a suspected recessive inheritance in this family, we prioritized biallelic variants. Two genes harbored multiple rare variants. After testing the variants in the parents, only one set of Sanger sequencing–validated compound heterozygous variants in KDR remained: KDR-p.(Gly347Trp) and KDR-p.(Gly537Arg) (Supplementary table 4). These variants were present in the two affected siblings in the compound heterozygous state, and were each inherited from an unaffected parent (Fig. 1c). The unaffected sister did not carry either KDR variant. KDR-p.(Gly347Trp) and KDR-p.(Gly537Arg) were not found in an internal data set of 390 controls of Moroccan descent or in any of the publicly available reference databases, including 3,065 individuals from North Africa and the Middle East in five population genetic data sets (Supplemental table 5). They were predicted to be deleterious by the in silico prediction tools SIFT and PolyPhen and had high CADD scores (35 and 34). The variants were located in the extracellular immunoglobulin-like domains 4 and 5 of VEGFR2, and the affected residues were evolutionarily conserved (Fig. 1d, e).

The only other gene harboring multiple variants was COL5A2. Although these variants were shared by the affected patients, they were proven to be in cis, inherited from the unaffected father (Supplementary table 4).

Kdr-p.(Gly535Arg) homozygous knock-in mice recapitulate the phenotype of Kdr+/− mice

Using CRISPR-Cas9 targeting, we generated knock-in mouse lines each carrying the mouse ortholog of the two variants found in the index family, i.e., Kdr-p.(Gly347Trp) (orthologous to KDR-p.(Gly347W) and compound heterozygotes KdrG535R/G535R mice. Scale bar is 0.5 cm. (b) Whole mount images E10.0 Kdr+/− and KdrG535R/G535R embryos. Normal vasculature (black arrows) as seen in Kdr+/− are not visible in KdrG535R/G535R embryos, indicating impaired endothelial development. Scale bar is 1 mm. (c) H&E stained sections of the developing heart of the Kdr+/− and KdrG535R/G535R embryos from (b), showing enlarged pericardial cavity in KdrG535R/G535R embryos (asterisk). Scale bar is 250 µm. (d) Anti-VEGFR2 stained single optical sections of endocardial cells from Kdr+/− and KdrG535R/G535R embryos. VEGFR2 is labeled green, WGA (cell membranes) is labeled red, and nuclei are labeled blue. Yellow arrowheads denote colocalization at the membrane of VEGFR2 and WGA and white arrowheads denote cytoplasmic VEGFR2. Scale bar is given in the figure. h heart, He head, pa pharyngeal arches, WGA wheat germ agglutinin.
[Gly345Trp]) and Kdr-p.(Gly535Arg) (orthologous to KDR-p. (Gly537Arg)), respectively. Heterozygous mice (Kdr<sup>G345W/+</sup> and Kdr<sup>G535R/+</sup>) were born at normal Mendelian ratios and their hearts were morphologically indistinguishable from control littermates. This was also the case for compound heterozygous mice (Kdr<sup>G345W/G535R</sup>) and mice homozygous for the Kdr<sup>-p.(Gly537Arg)</sup> variant (Kdr<sup>G347W/G537R</sup> (orthologous to KDR-p. (Gly537Arg))) (Fig. 2a and Supplementary table 6). However, we were unable to recover any mice homozygous for the Kdr-p.(Gly535Arg) variant (Kdr<sup>G535R/G535R</sup>) at birth (Supplementary table 7), suggesting that this variant may be embryonically lethal in the homozygous state. Previous studies have demonstrated that Kdr knockout animals die between embryonic day (E) 8.5 and E9.5 from severe vascular and hematopoietic abnormalities. Therefore, to determine whether Kdr<sup>G535R/G535R</sup> mice mimic null animals, we examined Kdr<sup>G535R/G535R</sup> embryos at E9.5. Although at this stage we found homozygous mice at normal Mendelian ratios (Supplementary table 7), homozygous mutants were smaller and paler than wild-type littermates with overall apparent necrosis and impaired endothelial development (Fig. 2b). Analysis of histological sections revealed an enlarged pericardial cavity in Kdr<sup>G535R/G535R</sup> embryos (Fig. 2c). Overall, the phenotype of Kdr<sup>G535R/G535R</sup> mice mimicked that of Kdr null mice, thus revealing that homozygosity of the Kdr-p.(Gly535Arg) variant has severe developmental consequences.

We next investigated the subcellular localization of VEGFR2 in endocardial tissue of E10.0 Kdr<sup>G535R/G535R</sup> and Kdr<sup>G535R/G535R</sup> mice. VEGFR2 signaling is highly active at this stage. Immunofluorescent staining of embryonic sections revealed VEGFR2 aggregation in the cytoplasm of endocardial cells in mutant embryos (Kdr<sup>G535R/G535R</sup> and Kdr<sup>G347W/G347W</sup>) that was largely absent in wild-type littermates (Fig. 2d), where VEGFR2 predominantly localized to cell membrane.

Familial KDR missense variants cause reduced VEGFR2 phosphorylation

In an effort to shed light on the biological mechanisms of the two KDR variants of the index family, we examined the phosphorylation of VEGFR2 at Tyr1175, one of its major phosphorylation sites and crucial for the recruitment of proteins in the signaling cascade downstream of VEGFR2. HEK 293T cells heterologously expressing the two identified variants or wild-type KDR (control) were stimulated with VEGF165. Western blot analysis on protein isolates using an antibody directed toward VEGFR2 showed two equally intense bands of the expected weight in cells expressing the wild-type VEGFR2. However, the lower band, representing unphosphorylated VEGFR2, was consistently and significantly more intense in cells expressing mutant VEGFR2, particularly those expressing KDR-p.(Gly537Arg) variant and those coexpressing KDR-p.(Gly345Trp) and KDR-p.(Gly537Arg). The latter condition models the compound heterozygous state of the two affected patients from the index family (Fig. 3a). Western blot analysis with p-VEGFR2 antibody detected one major band at the
expected weight (Fig. 3b), which had a significantly lower intensity for both variants expressed separately (40% and 80% reduction compared to wild-type for KDR-p.[Gly345Trp] and KDR-p. [Gly537Arg], respectively) and for the double mutant (60% reduction compared to wild type, \( P = 0.003 \); Fig. 3b). Similarly, western blot analysis of yolk sac cells from knock-in mouse embryos showed a seemingly modest reduction of p-VEGFR2 in Kdr-G535R/G535R mice, but a pronounced clear shift toward decreased relative phosphorylated VEGFR2 in Kdr-G347W/G347W, Kdr-G535R/G535R, and Kdr-G347W/G347R mice (Fig. 3c). Taken together, these data suggest that both variants cause decreased VEGFR2 phosphorylation at position Tyr1175. In line with the observed embryonic mortality and morphological phenotype of Kdrg347w/g347w mice, decreased VEGFR2 phosphorylation at position Tyr1175 was most pronounced for Kdr-p.[Gly537Arg] in HEK 293T cells and for the orthologous Kdr-p.[Gly535Arg] in mouse yolk sac cells.

Collectively, the genetic data from the family and the data obtained in mice and in vitro support a role for the two rare KDR missense variants in the index family, in which the functional effects of the KDR-p.[Gly537Arg] variant are more severe compared to those of KDR-p.[Gly345Trp]. A summary of the characteristics of both variants is given in Supplementary Table 8.

**KDR variants in different patient sets**

Based on the observation in the index family, we screened for coding variants and copy-number variants in KDR, using Sanger sequencing and qPCR respectively, in 82 patients with TOF who met at least one of the following criteria: (1) complex TOF, (2) positive family history of CHD, or (3) Moroccan descent. We identified a rare heterozygous missense variant (KDR-p.[Thr442Met], MAF gnomAD v2: 2 × 10⁻⁵) in one patient from the CONCOR biobank, with complex TOF, i.e., TOF and absent pulmonary valve syndrome (Supplementary Table 9). Information about ethnicity was not collected, and unfortunately both the patient and her parents were unavailable for follow-up or genetic testing. Like the KDR-p.[Gly537Arg] variant from the index family, this variant was also located in Ig-like domain 5 (Fig. 4). Similar to the variants from the index family, western blot analysis with the p-VEGFR2 antibody demonstrated significantly reduced p-VEGFR2 for KDR-p.[Thr442Met] compared to wild type (\( P < 0.001 \), Fig. 3d). No other coding variants or copy-number variants in KDR were detected in the remaining patients.

Through GeneMatcher we identified two more patients with complex TOF and a rare heterozygous variant in KDR, both PTVs: KDR-p.[R819*] and KDR-c.2614 + 1G>A (Fig. 4, Supplementary Table 10). One of those was de novo, while the other PTV was inherited from the father who had three strokes stemming from an underlying autoimmune disease (suggested Takayasu syndrome). Moreover, these patients also had extracardiac abnormalities (Supplementary Table 10).

In addition, we identified three patients with other types of CHD (with and without extracardiac abnormalities) and a heterozygous missense variant in KDR (de novo in two patients and inherited in the third), and two patients with a de novo KDR variant (one missense and one PTV, both heterozygous) had a noncardiac phenotype (Supplementary Table 10). This suggests that rare KDR variants might also be associated with other phenotypes.

**Table 1. Results burden test in patients with tetralogy of Fallot (TOF) (FAF < 0.01%).**

| Variant type | Protein domain | Cases: N variants/total N | Controls: N variants/total N | Fold enrichment | \( P \) value |
|--------------|----------------|--------------------------|-----------------------------|-----------------|--------------|
| PTV          | All            | 9/1,569                  | 7/56,728                    | 46              | \( 7 × 10^{-11} \) |
| Missense     | All            | 27/1,569                 | 791/56,721                  | 1.2             | 0.28         |
|              | All extracellular | 19/1,569              | 452/56,687                  | 1.5             | 0.08         |
|              | Ligand binding | 7/1,569                  | 116/56,791                  | 2.2             | 0.049        |
|              | Ig-like 4-7    | 8/1,569                  | 256/56,711                  | 1.1             | 0.70         |
|              | Protein kinase | 3/1,569                  | 122/56,722                  | 0.9             | 1            |
|              | All intracellular | 8/1,569                 | 339/56,722                  | 0.9             | 0.87         |

Statistically significant \( P \) values after correction for multiple testing are highlighted in bold (Fisher's exact test \( P < 0.007 \)).

**FAF filtering allele frequency, PTV protein-truncating variant.**

![Schematic diagram of VEGFR2 with all variants identified in tetralogy of Fallot (TOF) patients.](image-url)

**Fig. 4 Schematic diagram of VEGFR2 with all variants identified in tetralogy of Fallot (TOF) patients.** Location of rare KDR variants identified in patients with TOF (index family and 1,653 TOF probands). Variants are split into missense variants (black) and protein-truncating variants (PTVs) (red). Highlighted are the two variants identified in the index family, KDR-p.[Gly345Trp] and KDR-p.[Gly537Arg], and KDR-p. [Val219Ala] and KDR-p.[Thr442Met], that were functionally tested. Domains marked as I–VII represent the Ig-like domains 1 to 7. The transmembrane domain (gray box) separates the extracellular domain (left) of the intracellular domain (right). VEGFR2 subdomains are based on Roskoski\(^{20}\).

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|              | Protein kinase | 3/1,569                  | 122/56,722                  | 0.9             | 1            |
|              | All intracellular | 8/1,569                 | 339/56,722                  | 0.9             | 0.87         |
European descent drawn from a reference population (gnomAD-NFE v2.1). Rare KDR variants with a population maximum filtering allele frequency (FAF) < 0.01% (gnomAD v3.1) were considered. As expected, we saw no difference in the frequency of synonymous variants between cases and controls (Supplementary table 12). We identified a total of 36 protein altering variants, 9 PTVs, and 27 missense variants (Fig. 4 and Supplementary table 9), in 35 patients with TOF. One patient had two different KDR missense variants (KDR-p.[Gly23Ser] and KDR-p.[Thr442Met]), but we could not assess compound heterozygosity due to lack of parental samples. The remaining 34 patients carried heterozygous KDR variants. In total, we identified rare KDR variants in 2.3% of patients with TOF. Parental samples were available for 8 of 35 patients and showed all 8 variants were inherited. There was a significant excess (46-fold enrichment) of PTVs in TOF cases compared to gnomAD-NFE controls (9 PTVs in 1,569 TOF cases versus 7 PTVs in 56,699 fold enrichment) of PTVs in TOF cases compared to gnomAD-identiﬁed variants, 9 PTVs, and 27 missense variants (Fig. 4 and of synonymous variants between cases and controls (Supple-

DISCUSSION

We identiﬁed compound heterozygous rare missense variants in KDR in a family with two siblings affected by a severe, complex form of TOF. Subsequent studies conducted in knock-in mice and cell-based assays showed signiﬁcant diminished VEGFR2 autophosphorylation. In addition, the higher burden of PTVs in the 1,569 patients with TOF compared to controls further supports a loss-of-function mechanism. In total, 0.6% of patients with TOF in this study carried a PTV. The suggestive enrichment of rare missense variants in the extracellular domain of VEGFR2 in patients with TOF, together with the signiﬁcant reduction in VEGFR2 phosphorylation we showed for multiple missense variants from this domain, suggests that disruption of residues within the extracellular domain of VEGFR2 might play a role in the pathogenesis of TOF.

The patients from the index family in this study carried biallelic variants in KDR. The observations made in knock-in mice and in heterologous studies in vitro support a causal role for both missense variants and may suggest a recessive inheritance model. Although we identiﬁed one additional patient with two different rare KDR missense variants, compound heterozygosity could not be conﬁrmed in this patient due to lack of parental samples. KDR variants in all other patients in this study, as well as in a previously published study, were heterozygous. In some of these patients, the de novo occurrence of these variants provides support for dominant inheritance. On the other hand, the observation that some individuals inherited the putatively pathogenic variant from an unaffected parent points to reduced penetrance and a more complex inheritance, a phenomenon that can also not be excluded in the index family. Taken together, our data suggest that rare KDR variants may lead to TOF in the context of different inheritance paradigms that likely vary from monogenic to complex (oligogenic or polygenic).

We provide strong evidence that PTVs in KDR contribute to the pathogenesis of TOF. PTVs were approximately 45 times more prevalent among TOF cases compared to controls. In total we identiﬁed 11 PTVs in KDR in patients with TOF, not overlapping the two previously reported PTVs in patients with TOF.8 Although 8 of the 11 identiﬁed PTVs (73%) were located in the intracellular domain, these numbers are too small to make any statement about nonrandom distribution in TOF cases. Interestingly, we did not ﬁnd any PTVs among a set of 2,312 patients with CHDs other than TOF, which may suggest that loss of function of this gene speciﬁcally predisposes to TOF within the spectrum of CHD. Of note, PTVs in KDR were recently also reported in patients with pulmonary arterial hypertension (PAH).19,20 although none of the PAH cases were reported to have CHD. What determines why some PTVs lead to TOF, while others might cause PAH, remains unclear.

Though not statistically signiﬁcant, we observed a higher proportion of rare KDR missense variants in the extracellular domain of VEGFR2 in patients with TOF. One of these variants, KDR-p.(Val219Ala), was found in multiple patients and showed a marked reduced effect on VEGFR2 phosphorylation. This was in line with the KDR variants of the index family, and of a patient with TOF and absent pulmonary valve syndrome (KDR-p.[Thr442Met]), that were all located within the extracellular domain and also exhibited signiﬁcantly reduced VEGFR2 phosphorylation. Similarly, missense variants in FLT4, encoding for VEGFR3, previously reported in patients with TOF, were primarily located in the extracellular domain.6,10 Clearly, larger studies are needed to conﬁrm whether the extracellular domain is indeed speciﬁcally enriched in rare missense variants, as well as to explore differences in phenotype based on variant location in the protein. In addition, future studies should also assess whether there are phenotypic differences between patients with amorphic versus hypomorphic KDR variants.

The importance of VEGFR2 in heart development has long been established. While Kdr KO mice die early during development, conditional deletion of mouse Kdr in the endothelium (using an endothelial-speciﬁc Cre driver) causes embryonic lethality at E9.5–E10.5 associated with cardiac defects, including hypoplasia of the outﬂow tract (OFT) and the right ventricle, and loss of the endocardium,21 highlighting the role of VEGFR2 in OFT development. The second heart field (SHF) is essential for formation of the OFT and right ventricle,22 both regions of the heart affected in TOF. Conditional deletion of Kdr in the Isl1 lineages, which includes the SHF, while showing preserved endocardium, leads to embryonic lethality at E14.5,23 supporting an important role of VEGFR2 in cardiogenesis independent from its role in the endocardium. The role of VEGFR2 in the SHF is further highlighted by the presence of Vegfr2 expression in the pharyngeal mesoderm at E8.5 and, at later stages, in (parts) of the SHF.25 Moreover, it was shown that the Tbx1 transcription factor, essential during OFT development, affects Vegfr2 expression in the SHF in vivo in mouse embryos and that Tbx1 favors a cardiac fate in Vegfr2 expressing cells.25 Given the above, we hypothesize that altered Vegfr2 expression and/or function within the SHF could directly or indirectly contribute to malformation of the OFT, and therefore TOF.
In an effort to provide evidence for causality of missense variants in KDR and determine their mechanism of pathogenicity, we engineered knock-in mouse lines harboring orthologues of the two variants identified in the index family. Contrary to the index family, where the disease phenotype was presumed to result from biallelic inheritance, compound heterozygous knock-in mice (i.e., KdrG347W/G535R) were phenotypically normal. The lack of phenotype in the double heterozygous knock-in mice could be due to genetic background effects, a phenomenon that is firmly established for CHD.26 Although KdrG347W/G347W exhibited no morphological abnormalities, mice homozygous for the orthologous variant to the familial KDR-p.(Gly537Arg) variant (KdrG347R/G347R) died during embryonic development and recapitulate the phenotype of constitutive Kdr knockout mice,16 underscoring the causality and severity of this variant. The varying severity of these two variants in mice in the homozygous state is reflected by the observed differences in severity in their Tyr1175 (p-VEGFR2) phosphorylation defect. Similar to the phosphorylation data in the mouse, in HEK 293T cells we observed a marked reduction in p-VEGFR2 in cells co-expressing the two variants or expressing KDR-p.(Gly537Arg) alone, while a milder decrease was seen in cells expressing the KDR-p.(Gly345Trp) variant. In aggregate, these data and the co-segregation data in the family support the concept that both alleles may be necessary for the development of the phenotype in the family, but contribute differentially to disease susceptibility, with the KDR-p.(Gly537Arg) variant having a larger effect than the KDR-p.(Gly345Trp) variant.

The extracellular domain of VEGFR2 is a critical part of the protein as it harbors the VEGF binding domain, and initiates receptor dimerization upon ligand binding.1 Furthermore, homotypic receptor–receptor contacts between Ig-like domains 4 and 7 further stabilize these VEGFR2 dimers and are essential for the exact positioning of the intracellular kinase domains,27,28 which institute protein kinase activation, trans-autophosphorylation (among others on Tyr1175), and initiation of signaling pathways. We suggest that the tested missense variants in the extracellular domain, i.e., KDR-p.(Gly345Trp), KDR-p.(Gly537Arg), KDR-p.(Val1219Ala), and KDR-p.(Thr442Met), disturb this sequence of events, likely leading to diminished autophosphorylation and receptor activation. At the same time, in normal situations, ligand induced VEGFR2 activation stimulates the recycling of the intracellular VEGFR2 pool,29 as well as exit of newly synthesized VEGFR2 from the Golgi,30 thereby increasing the fraction of VEGFR2 on the plasma membrane. The intracellular accumulation of VEGFR2 that we observed for the KDR-p.(Gly537Arg) variant could indicate that this variant may interfere with this process. In turn, as receptors in intracellular vesicles are not accessible for VEGF, abnormal accumulation might further reduce the VEGFR2 phosphorylation levels. Regarding the KDR PTVs, we expect most if not all PTVs detected in TOF patients to result in nonsense-mediated decay (based on their location) and haploinsufficiency. We hypothesize that such reduced levels of VEGFR2 will impact on the total absolute amount of autophosphorylated VEGFR2, ultimately affecting proper function and development. Indeed phosphorylation of VEGFR2 at Tyr1175 has been shown to be essential for endothelial and hematopoietic development during embryogenesis.31 Yet, despite our finding of reduced autophosphorylation as a consequence of genetic variation in KDR, the exact involvement of reduced autophosphorylation in pathogenesis of TOF remains to be studied. While VEGFR2 signaling is extremely complex, and more than half a dozen pathways are recognized,19 a possible link between malfunctioning VEGFR2 phosphorylation and CHD comes from the knowledge that Tyr1175 phosphorylation is important in activation of the PLCγ-ERK1/2 pathway17,18 and that disturbed ERK1/2 signaling contributes to cardiac defects that comprise TOF in vivo.5,23 Future studies are needed to address the exact downstream pathways affected by decreased VEGFR2 Tyr1175 phosphorylation.

Limitations
Although we showed that the KDR variants in the index family are absent from publicly available reference data sets and 390 internal Moroccan controls, larger region-specific population genetic data sets will be required to fully confirm the rarity of the variants detected in Moroccan patients. In this study we only focused on rare variants in KDR and did not explore a multigenic inheritance. We did not functionally test all variants identified in patients and are therefore not able to conclude on their pathogenicity. We used individuals from the gnomAD collection as a control data set in the burden analysis. Although there are some inevitable limitations to this approach, it has been shown to be successful in other inherited cardiac disorders.34

Conclusion
In conclusion, our data supports a role for rare KDR variants in pathogenesis of TOF through a loss-of-function mechanism. The total yield of rare KDR (VEGFR2) variants in TOF patients in this study (2.3%) is comparable to the previously reported yield of rare FLT4 (VEGFR3) variants in patients with TOF. Taken together, the findings in this study shed light on the role of VEGF signaling in TOF and justify consideration of KDR screening in TOF patients in a clinical diagnostic setting.

DATA AVAILABILITY
Available upon request.

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REFERENCES
1. Bailliard, F. & Anderson, R. H. Tetralogy of Fallot. Orphanet J. Rare Dis. 4, 2 (2009).
2. Egbe, A., Ho, D., Lee, S., Uppu, S. & Srivastava, S. Prevalence of congenital anomalies in newborns with congenital heart disease diagnosis. Ann. Pediatr. Cardiol. 7, 86 (2014).
3. Burn, J. et al. Recurrence risks in offspring of adults with major heart defects: results from first cohort of British collaborative study. Lancet. 351, 311–316 (1998).
4. Peyvandi, S. et al. Risk of congenital heart disease in relatives of probands with aconotrinal cardiac defects: an evaluation of 1,620 families. Am. J. Med. Genet. A. 164, 1490–1495 (2014).
5. Goldmuntz, E. et al. Frequency of 22q11 deletions in patients with conotruncal defects. J. Am. Coll. Cardiol. 32, 492–498 (1998).
6. Yang, Y. Q. et al. GATA4 loss-of-function mutations underlie familial tetralogy of Fallot. Hum. Mutat. 34, 1662–1671 (2013).
7. Goldmuntz, E., Geiger, E. & Benson, D. W. NIX2.5 mutations in patients with tetralogy of fallot. Circulation. 104, 2565–2568 (2001).
8. Page, D. J. et al. Whole exome sequencing reveals the major genetic contributors to non-syndromic tetralogy of Fallot. Circ. Res. 124, 553–563 (2018).
9. Jin, S. C. et al. Contribution of rare inherited and de novo variants in 2,871 congenital heart disease probands. Nat. Genet. 49, 1593–1601 (2017).
10. Reuter, M. S. et al. Haplosufficiency of vascular endothelial growth factor related signaling genes is associated with tetralogy of Fallot. Genet. Med. 21, 1001–1007 (2018).
11. Holmes, K., Roberts, O. L., Thomas, A. M. & Cross, M. J. Vascular endothelial growth factor receptor-2: structure, function, intracellular signalling and therapeutic inhibition. Cell. Signal. 19, 2003–2012 (2007).
12. Sobrieira, N., Schiettecatte, F., Valle, D. & Hamosh, A. GeneMatcher: a matching tool for connecting investigators with an interest in the same gene. Hum. Mutat. 36, 928–930 (2015).
13. Sifrim, A. et al. Distinct genetic architectures for syndromic and nonsyndromic congenital heart defects identified by exome sequencing. Nat. Genet. 48, 1060–1065 (2016).
14. Homzy, J. et al. De novo mutations in congenital heart disease with neurodevelopmental and other congenital anomalies. Science. 350, 1262–1266 (2015).
15. Karczewski, K. J. et al. The mutational constraint spectrum quantified from variation in 141,456 humans. Nature. 581, 434–443 (2020).
16. Chalaby, F. E. et al. Failure of blood-island formation and vasculogenesis in Flik-1 deficient mice. Nature. 376, 62–66 (1995).
17. Takahashi, T., Yamaguchi, S., Chida, K. & Shibuya, M. A single autophosphorylation site on KDR/Flik-1 is essential for VEGF-A-dependent activation of PLC-gamma and DNA synthesis in vascular endothelial cells. EMBO J. 20, 2768–2778 (2001).
18. Kroll, J. & Weltenberger, J. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. J. Biol. Chem. 272, 32521–32527 (1997).
19. Koch, S., Tugues, S., Li, X., Guanandi, L. & Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. Biochem. J. 437, 169–183 (2011).
20. Roskoski, R. VEGF receptor protein-tyrosine kinases: structure and regulation. Biochem. Biophys. Res. Commun. 375, 287–291 (2008).
21. Graf, S. et al. Identification of rare sequence variation underlying heritable pulmonary arterial hypertension. Nat. Commun. 9, 1–16 (2018).
22. Eyries, M. et al. Familial pulmonary arterial hypertension by KDR heterozygous loss of function. Eur. Respir. J. 55, 1902165 (2020).
23. Milgrom-Hoffman, M. et al. The heart endocardium is derived from vascular endothelial progenitors. Development. 138, 4777–4787 (2011).
24. Xu, H. et al. Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. Development. 131, 3217–3227 (2004).
25. Lania, G., Ferrentino, R. & Baldini, A. TBX1 represses vegfr2 gene expression and enhances the cardiac fate of VEGFR2− cells. PLoS One. 10, e0138525 (2015).
26. Rajagopal, S. et al. Spectrum of heart disease associated with murine and human GATA4 mutation. J. Biol. Chem. 4787 (2011).
27. Hyde, C. A. C. et al. Targeting extracellular domains D4 and D7 of vascular endothelial growth factor receptor 2 reveals allosteric receptor regulatory sites. Mol. Cell. Biol. 32, 3802–3813 (2012).
28. Gampel, A. et al. VEGF regulates the mobilization of VEGFR2/KDR from an intracellular endothelial storage compartment. Blood. 108, 2624–2631 (2006).
29. Manicam, V. et al. Regulation of vascular endothelial growth factor receptor 2 trafficking and angiogenesis by Golgi localized t-SNARE syntaxin 6. Blood. 117, 1425–1435 (2011).
30. Sakurai, Y., Ohgimoto, K., Kataoka, Y., Yoshida, N. & Shibuya, M. Essential role of Flik-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. J. Biol. Chem. 23527 (1997).
31. Sakurai, Y., Ohgimoto, K., Kataoka, Y., Yoshida, N. & Shibuya, M. Essential role of Flik-1 (VEGF receptor 2) tyrosine residue 1173 in vasculogenesis in mice. Proc. Natl. Acad. Sci. U. S. A. 102, 1076–1081 (2005).
32. Araki, T. et al. Mouse model of Noonan syndrome reveals cell type- and gene dosage-dependent effects of Ptpn11 mutation. Nat. Med. 10, 849–857 (2004).
33. Krenz, M., Yutzey, K. E. & Robbins, J. Noonan syndrome mutation Q79R in Shp2 increases proliferation of valve primordia mesenchymal cells via extracellular signal-regulated kinase 1/2 signaling. Circ. Res. 97, 813–820 (2005).
34. Mazzarotto, F. et al. Reevaluating the genetic contribution of monogenic dilated cardiomyopathy. Circulation. 141, 387–398 (2020).

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ETHICS DECLARATION

Human subjects: Most participants were ascertained via research studies. These individuals gave consent to their research participation. This study was approved by the Amsterdam University Medical Center Research Ethics Committee, the Northern and Yorkshire Research Ethics Committee, the Hospital for Sick Children research ethics board and the ethics committee of the Charité Universitätsmedizin Berlin (#EA2/131/10). For samples from the CONCOR registry (https://concor.net/en/) informed consent was obtained from all patients for collection of samples and data for research including information about reporting of individual results and incidental findings (PMID 16121765). For samples from the Heart Centre Biobank Registry (https://theheartcentrebiobank.com/) informed consent was obtained from all patients/parents/legal guardians for collection of samples and data for research including sharing with internal and external researchers (PMID 23045559). The use of samples from the German Competence Network for Congenital Heart Defects (CNCHD) (http://www.kompetenznetz-ahf.de/en/researchers/join-our-research/national-register-biobank/) is based on a broad consent according to the principles of the Declaration of Helsinki (PMID 27132144). Participants who were identified via clinical diagnostics procedures (only GeneMatcher individuals) gave clinical consent for testing using standard forms used locally at each site, and their permission for anonymized inclusion in this publication. Mouse studies: Animal care and experiments were in accordance with guidelines from the European Union, the Dutch government and the Amsterdam University Medical Center (UMC) and were approved by the Animal Experimental Committee of the Amsterdam UMC.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information

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Correspondence and requests for materials should be addressed to A.V.P. or C.R.B.

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Marc-Phillip Hitz31, Hashim Abdul-Khaliq32, Felix Berger33, Ingo Dähnert34, Sven Dittrich35, Anselm Uebing36 and Brigitte Stiller37

31Universitätsklinikum Schleswig-Holstein Kiel, Kiel, Germany. 32Universitätsklinikum des Saarlandes, Homburg, Germany. 33Deutsches Herzzentrum Berlin, Berlin, Germany. 34Herzzentrum Leipzig, Leipzig, Germany. 35Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany. 36Universitätsklinikum Schleswig-Holstein, Kiel, Germany. 37Universitäts-Herzzentrum Freiburg Bad Krozingen, Freiburg, Germany.