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MAP2K1 Mutation in Zebrafish Endothelial Cells Causes Arteriovenous Shunts Preventable by MEK Inhibition

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Abstract

Objectives: Arteriovenous malformation (AVM) is a congenital lesion with a nidus of irregular blood vessels connecting arteries to veins instead of a normal capillary bed. Somatic *MAP2K1* activating mutations in endothelial cells cause extracranial AVM. The purpose of this study was to create a *MAP2K1* AVM animal model using zebrafish and to test pharmacotherapy.

Methods: Single-cell *casper* Tg(*gata1a*:DsRed) zebrafish embryos were injected with plasmid DNA (control [pTol2-*Fli*:GFP]; mutant [pTol2-*Fli*:GFP-*kdrl*:MAP2K1^{K57N}]) and Tol2 transposase mRNA to mosaically express activated MAP2K1 in endothelial cells. Two cohorts of fish were examined: group 1 (n = 161) established phenotypes and group 2 (n = 126) tested MEK inhibition. Blood flow was visualized using DsRed fluorescence of erythrocytes. Embryos were imaged 72 hours postfertilization.

Results: Group 1 exhibited abnormal arteriovenous shunts in 58 of 96 (60%) embryos expressing MAP2K1^{K57N} in endothelial cells. Shunts occurred between the proximal aorta and common cardinal vein (n = 39; 67%) or between the major artery and vein within the trunk or tail (n = 19; 33%). Shunts were not present in control zebrafish (n = 65). MEK inhibition reduced shunt frequency caused by endothelial MAP2K1^{K57N} expression in group 2 from 84% to 55% (0.2 μM) or 25% (0.4 μM) (P = .006).

Conclusions: Zebrafish endothelial cells expressing mutant *MAP2K1* form abnormal arteriovenous shunts supporting the causality of the variant in human AVMs. MEK inhibition reduced shunt formation validating its potential efficacy as a pharmacotherapeutic option for AVM. This zebrafish model may be used for further study of the etiopathogenesis of AVM as well as to test drugs.

Keywords: arteriovenous malformation, AVM, MAP2K1, MEK, shunt, trametinib, vascular, zebrafish

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Introduction

Arteriovenous malformation (AVM) is a sporadic congenital vascular anomaly that is present at birth and progresses over time. Lesions contain a nidus of abnormal blood vessels connecting arteries directly to veins without a normal capillary bed. Patients suffer deformity, ulceration, bleeding, pain, and occasionally congestive heart failure. Treatment includes embolization or resection, but recurrence is common and patients rarely are cured. Our group showed that extracranial AVMs contain an activating somatic mutation in *MAP2K1* isolated to endothelial cells.[1] The goals of this study were to: (1) determine if the *MAP2K1* mutation was sufficient to cause arteriovenous shunts in vivo, (2) establish a *MAP2K1*-mutant animal model of AVM to further investigate the etiopathogenesis of the lesion, and (3) develop an in vivo assay to test novel pharmacotherapies for AVM.

Methods

Creation of MAP2K1 zebrafish

Experiments were performed in accordance with Boston Children's Hospital Institutional Animal Care and Use Committee. Zebrafish were maintained under standard conditions.[2] A description of the husbandry and environmental

conditions is available at https://www.protocols.io/ (dx.doi.org/10.17504/protocols.io.mrjc54n). Control (pTol2-*fli1ep:egfp*) and mutant (pTol2-*fli1ep:egfp-kdrl*:MAP-2K1^{K57N}) plasmids were synthesized. Plasmid JDW 770 (pTol2-*kdrl*:EGFP-hsKRAS4B-G12V-ac/Y) was obtained from Addgene.[3] The *KRAS* mutation was replaced with the *MAP2K1* K57N mutation under control of the endothelial-specific *kdrl* promoter.[4] A *fli1ep:egfp* reporter[5] was substituted using in-fusion cloning. Because the *MAP2K1* mutation in human AVM is isolated to the endothelial cell, the *kdrl* promoter was chosen to express MAP2K1^{K57N} in zebrafish endothelial cells. Plasmids were co-injected with Tol2 *transposase* mRNA at the single-cell stage.

All experiments were performed in transgenic *Tg(gata1a:DsRed)^{sd2}* fish (Leonard Zon ZDB-ALT-051223-6) in the casper background.[6] *Tg(gata1a:DsRed)^{sd2}* fish were selected because red blood cells fluoresce red allowing for visualization of blood flow. Embryos were dechorinated using forceps for imaging before hatching and housed at 28°C in E3 medium. Single-cell stage embryos were injected with 1 nL of transgenesis mixture (100 ng/μL of either control (pTol2-*fli1ep:egfp*) or mutant (pTol2-*fli1ep:egfp-kdrl*:MAP-2K1^{K57N} plasmid DNA) + 20 ng/μL Tol2 *transposase* mRNA. To establish the phenotype of injected fish 161 embryos were examined (group 1).

Treatment with MEK inhibition

To determine the effects of MEK inhibition on shunt formation, a second cohort of 126 fish was used (group 2). Trametinib, a MAP2K1 inhibitor[7] (Cat. No. HY-10999; MedChemExpress, Monmouth Junction, NJ) was dissolved in DMSO and heated to 42°C. The solution then was cooled to room temperature and added to E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, 5% methylene blue). Three groups were studied: (1) DMSO control (1 μM), (2) 0.2 μM trametinib, and (3) 0.4 μM trametinib. The solutions were added to E3 media 28 hours post-fertilization. Trametinib-treated E3 media was replaced at 24 hours.

Phenotype analysis

Embryos were anesthetized with 0.4 mg/mL tricaine, embedded in 1% low-melt agarose (Biobasic, Markham ON, Canada), and examined using a Zeiss LSM 800 confocal microscope with Zen Blue (version 2.5). Blood flow was assessed using a Nikon SMZ18 fluorescence microscope. Non-living fish, those with failed transgenesis, or embryos with gross morphological changes excluding the vasculature were not analyzed. Fish with evidence of transgenesis were assessed for changes in blood flow 72 hours post-fertilization by visualizing fluorescence in red blood cells and/or blood flow using light microscopy. *Tg(fli1ep:egfp)*-injected control fish also were examined if evidence of transgenesis was present. The investigator was blinded to treatment group during phenotype quantification. Statistical significance was determined using 2-sample *t* test for phenotype quantification and 1-way analysis of variance with Tukey multiple comparisons for drug treatment.

Results

Control zebrafish exhibited normal circulatory patterns with clearly separated arteries and veins in the trunk and head with no evidence of arteriovenous shunts (n = 65; Figures 1A, 2A, B). In contrast, embryos expressing endothelial MAP2K1^{K57N} exhibited 2 major shunt phenotypes (group 1). The most common phenotype was a proximal shunt with a terminal connection between a branch of the lateral dorsal aorta and the adjacent common cardinal vein with minimal or no distal blood flow (39/96 embryos; 40%; Figures 1B, 2C, 3). The second major phenotype was a direct abnormal connection between the major artery and vein within the trunk or tail (19/96 embryos, 20%). These shunts had either: (1) a direct fistula with intact distal blood flow (Figures 1C, 2D, E) or (2) a terminal connection with minimal or no distal blood flow (Figures 1D, 2F). Several endothelial cells lining the shunt expressed high levels of the marker transgene confirming shunts contained MAP2K1^{K57N}-expressing endothelial cells (Figure 2E). Endothelial cells expressing the transgene also were identified in areas of phenotypically

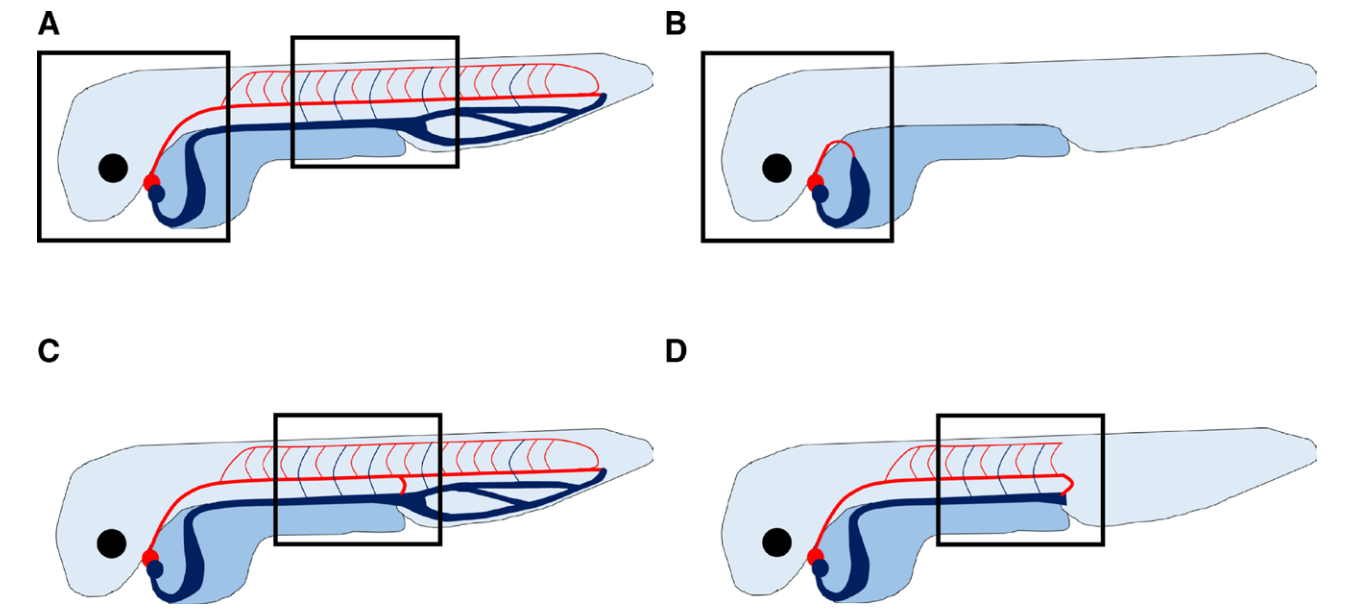


Figure 1. Schematic of observed phenotypes. A, Normal zebrafish vasculature, (B) proximal shunt phenotype observed in embryos expressing endothelial MAP2K1^{K57N}. C, Distal fistula phenotype observed in MAP2K1^{K57N}-expressing embryos. D, Terminal connection with minimal or no distal flow observed in some MAP2K1^{K57N} embryos. Boxes indicate area of embryo represented by confocal images in Figure 2.

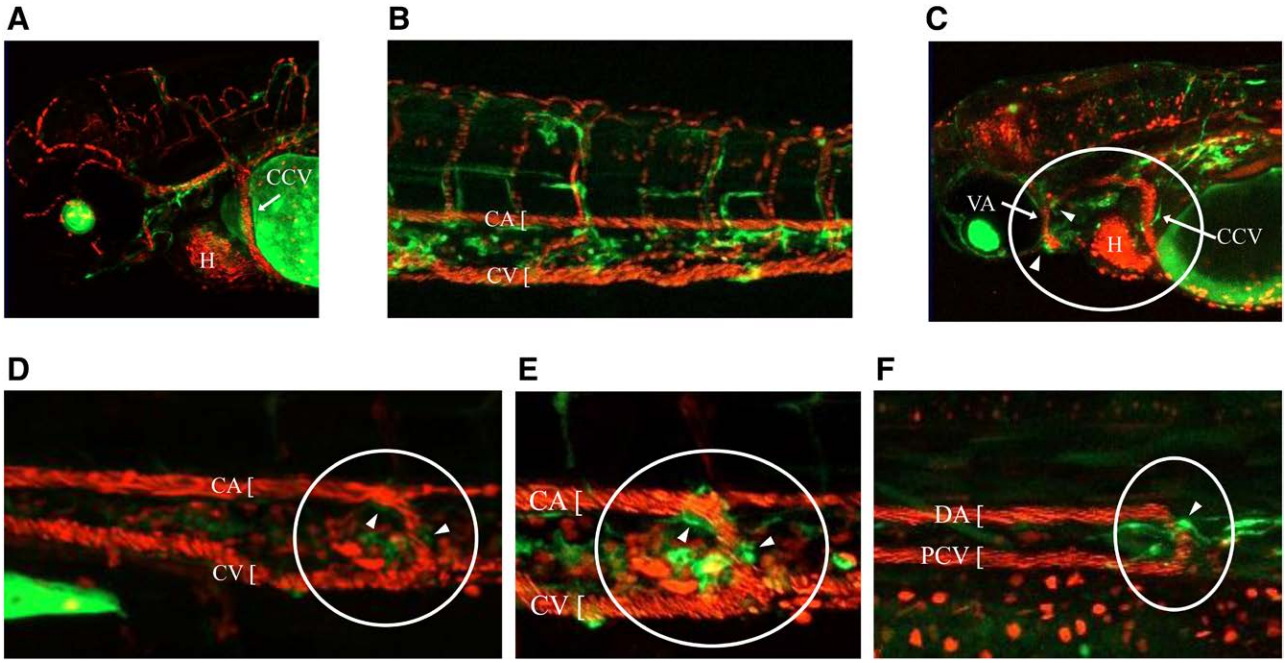


Figure 2. Expression of activated MAP2K1 in zebrafish endothelial cells causes arteriovenous shunts. A and B, Confocal images of normal rostral (A) and tail (B) vasculature in zebrafish injected with control GFP plasmid. C, Confocal image of MAP2K1^{K57N} embryo showing an abnormal proximal shunt between the VA and CCV. D, Confocal image of MAP2K1^{K57N} embryo with fistula between the CA and CV at 10×. E, Confocal image at 20× demonstrates the fistula contains mutant endothelial cells expressing high levels of the marker transgene. F, Confocal image of MAP2K1^{K57N} embryo with terminal connection occurring between the DA and PCV. Arteriovenous shunts are identified by white circles. Arrowheads indicate transgene-expressing endothelial cells. CA, CCV, CV, DA, H, PCV, VA. CA indicates caudal artery; CCV, common cardinal vein; CV, caudal vein; DA, dorsal artery; H, heart; PCV, posterior cardinal vein; VA, ventral aorta.

normal vasculature. MEK inhibition led to a statistically significant dose-dependent reduction of shunt formation in embryos expressing endothelial MAP2K1^{K57N} from 84% to 55% (0.2 μM) and 25% (0.4 μM) (P = 0.006; Figure 4).

Discussion

Expression of mutant *MAP2K1* (K57N) in zebrafish endothelial cells resulted in abnormal arteriovenous shunts, supporting the causality of this variant in human AVMs. Our model shared some similarities and differences with other investigators who have created zebrafish with RAS/MAPK signaling pathway variants. Al-Olabi et al[8] demonstrated postzygotic mosaic expression of mutant BRAF (V600E) and MAP2K1 (Q58del) caused disordered formation of the caudal vein plexus and impeded blood flow. A modest improvement in blood flow was seen after treatment with a RAF inhibitor. Bell et al[9] created a MAP2K1 (K57N)

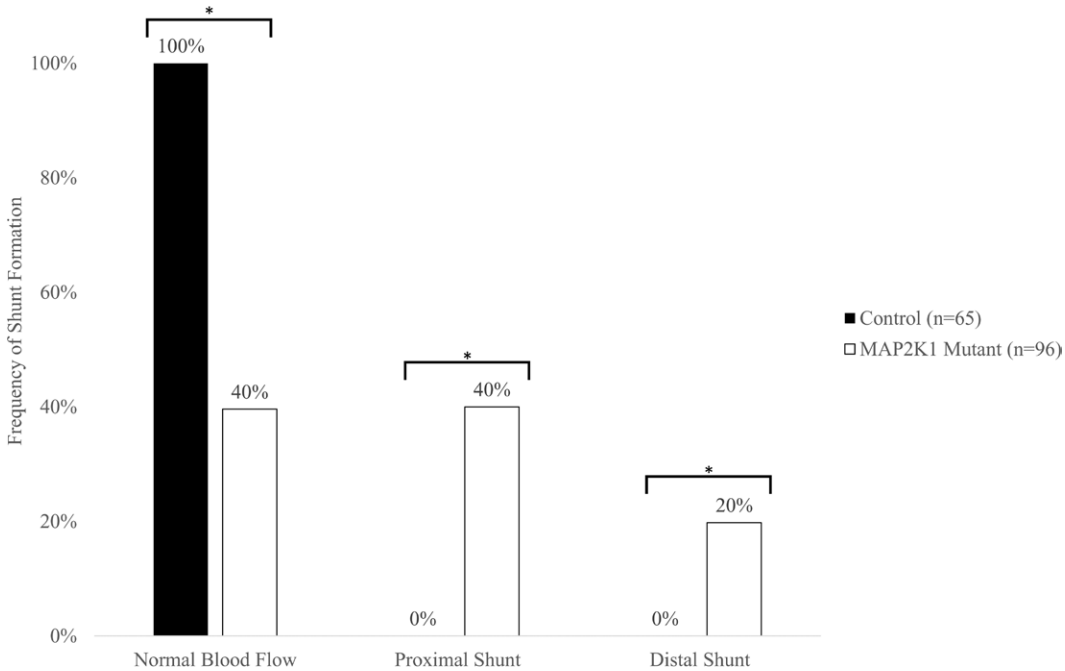


Figure 3. Group 1 MAP2K1 mutant zebrafish phenotype quantification (control n = 65, *MAP2K1* mutant n = 96). Statistical significance was determined by 2-sample *t* test. *P < .05.

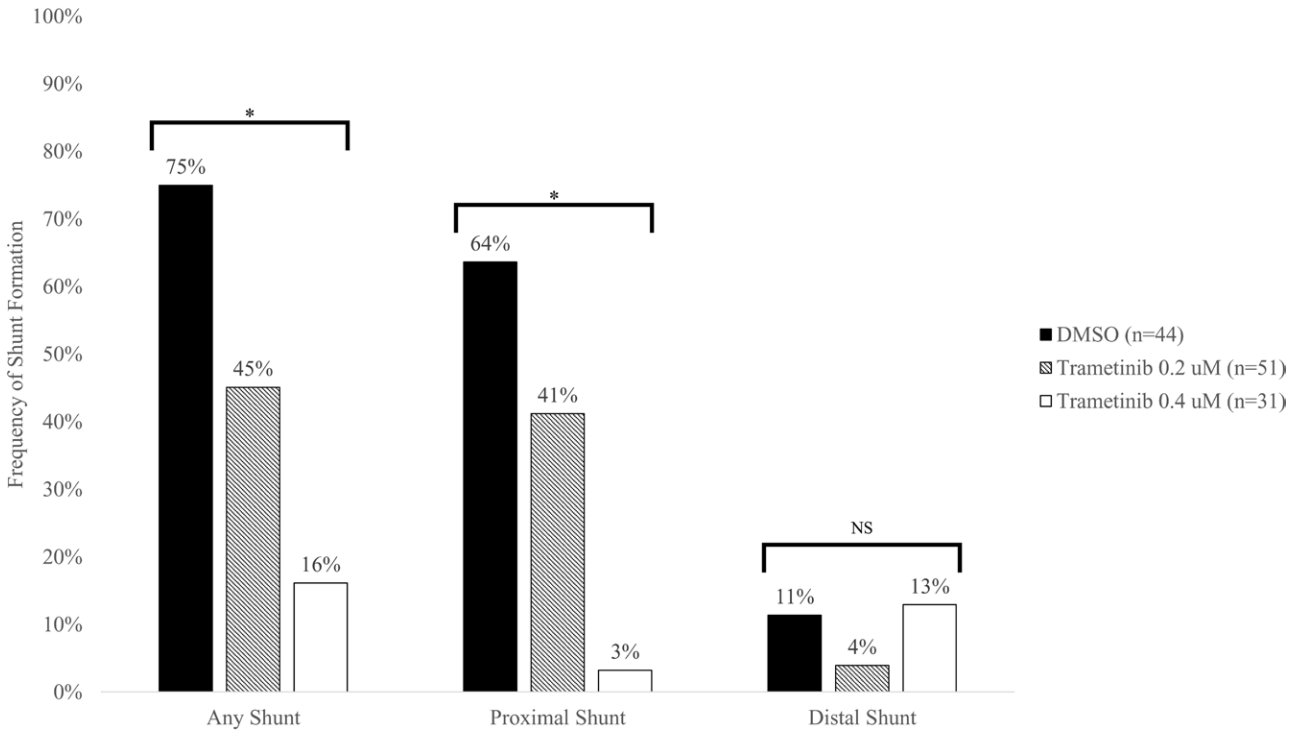


Figure 4. Group 2 trametinib treatment of MAP2K1 mutant zebrafish reduced the frequency of shunt formation (dimethyl sulfoxide n = 44, trametinib 0.2 μM n = 51, trametinib 0.4 μM n = 31). Statistical significance was determined by 1-way analysis of variance with Tukey multiple comparisons. *$P < .05$; NS indicates not significant.

fish as a comparison group while evaluating mutations in TEK. These fish were made using the Gal4-UAS system and formed abnormal arteriovenous connections at 30–48 hours postfertilization in the distal tail. Fish et al[3] most closely mirrored our phenotype injecting zebrafish with *kdrl*:EGFP-KRAS (G12V); 48% had dorsal artery and cardinal vein shunts reduced with MEK inhibition using SL327.

Although MEK inhibition caused a dose-dependent reduction in total and proximal shunt formation, it did not significantly lower the frequency of distal shunts. This may have occurred because distal shunt formation was less common and thus underpowered to observe an effect. Clinically, MEK inhibition may slow AVM growth by preventing progressive shunt formation. MEK inhibition also may reduce recurrent shunt formation following excision or embolization of AVMs. The ability of MEK inhibition to treat established shunts remains unclear.

Shunts in our fish contained a combination of mutant and wild-type endothelial cells. Mutant endothelial cells were present in areas of phenotypically normal vessel development, indicating mutant endothelial cells are necessary but not sufficient to form shunts. Shunts may develop from a cell nonautonomous mechanism which occurs with human AVM growth.[10] This could be tested using time-lapse confocal imaging of mutant fish tracking differences in which mutant endothelial cells form shunts. This technique also could be used to examine if shunt formation originates from an arterial or venous endothelial cell.

Improved treatment options for patients with AVM are desperately needed and MEK inhibition appears to be a promising strategy in humans.[11,12] Clinical trials with MEK inhibitors are ongoing in Europe and the United States. Our finding that MEK inhibition reduced shunt formation in zebrafish embryos with endothelial-expressed MAP2K1^{K57N} supports the use of MEK inhibitors for patients with AVM. This zebrafish model allows for further testing of novel pharmacotherapeutic options.

References

1. Couto JA, Huang AY, Konczyk DJ, et al. Somatic MAP2K1 mutations are associated with extracranial arteriovenous malformation. Am J Hum Genet. 2017;100:546–554.
2. Westerfield M. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio Rerio). 4th ed. Eugene: University of Oregon Press; 2000.
3. Fish JE, Wythe JD, Perfecto C, et al. Somatic gain of KRAS function in the endothelium is sufficient to cause vascular malformations that require MEK but not PI3K signaling. Circ Res. 2020;127:727–743.
4. Covassin LD, Siekmann AF, Kacergis MC, et al. A genetic screen for vascular mutants in zebrafish reveals dynamic roles for Vegf/Plcg1 signaling during artery development. Dev Biol. 2009;329:212–226.
5. Villefranc JA, Amigo J, Lawson ND. Gateway compatible vectors for analysis of gene function in the zebrafish. Dev Dyn. 2007;236:3077–3087.
6. White RM, Sessa A, Burke C, et al. Transparent adult Zebrafish as a tool for in vivo transplantation analysis. Cell Stem Cell. 2008;2:183–189.
7. Smits PJ, Konczyk DJ, Sudduth CL, Goss JA, Greene AK. Endothelial MAP2K1 mutations in arteriovenous malformation activate the RAS/MAPK pathway. Biochem Biophys Res Commun. 2020;529:450–454.
8. Al-Olabi L, Polubothu S, Dowsett K, et al. Mosaic RAS/MAPK variants cause sporadic vascular malformations which respond to targeted therapy. J Clin Invest. 2018;128:1496–1508.
9. Bell LM, Holm A, Matysiak U, et al. Functional assessment of two variants of unknown significance in TEK by endothelium-specific expression in Zebrafish embryos. Hum Mol Genet. 2021;31:10–17.
10. Konczyk DJ, Goss JA, Smits PJ, Sudduth CL, Al-Ibraheemi A, Greene AK. Arteriovenous malformation MAP2K1 mutation causes local cartilage overgrowth by a cell-non autonomous mechanism. Sci Rep. 2020;10:4428.
11. Lekwuttikarn R, Lim YH, Admani S, Choate KA, Teng JMC. Genotype-guided medical treatment of an arteriovenous malformation in a child. JAMA Dermatol. 2019;155:256–257.
12. Edwards EA, Phelps AS, Cooke D, et al. Monitoring arteriovenous malformation response to genotype-targeted therapy. Pediatrics. 2020;146:e20193206.