Manuscript Title: Venous Malformation: from causative *TIE2* mutations to targeted therapy.

SUPPLEMENTARY DATA:

DETAILED MATERIALS AND METHODS

Cell Culture

HUVECs were generously provided by Dr. F. W. Luscinskas at Brigham and Women's Hospital, Boston, MA, USA. Full-length TIE2-WT or TIE2-L914F were cloned into pMXs vector (gift from T. Kitamura, University of Tokyo, Japan). Packaging cell line 293-GPG VSV-G (1) was transfected for retrovirus production with Fugene 6 (Roche). Retrovirally transfected HUVEC-lines were expanded in culture on attachment factor solution-treated (Cell Applications, Inc.) tissue culture dishes and fed with Endothelial Cell Growth Medium (Cell Applications, Inc.) /10% fetal bovine serum (HyClone).

In Vivo Murine Model of VM

Experiments were carried out by implanting $2.5x10^6$ HUVECs (TIE2-WT or TIE2-L914F) in immune-deficient mice, as in our previously described murine model of infantile hemangioma (2). In brief, cells were counted, suspended in 200 µl of Matrigel™ (BD Bioscience) and injected sub-cutaneously (s.c.) on the lower back of 6-7 weeks old male athymic nu/nu mice (Massachusetts General Hospital, Boston, MA) (n =8-10/group). The care and treatment of experimental animals were in accordance with institutional guidelines. Area of lesions (mm^2) was measured with caliper every 2 or 5 days. Weight was monitored to assess health status of the mice.

For drug treatment studies, HUVEC-TIE2-L914F were incubated for 48 hours in vitro with 5 µM TIE2 tyrosine kinase inhibitor (TIE2-TKI) 4-(6-Methoxy-2-naphthyl)-2-(4-methylsulfinylphenyl)-5-(4-

pyridyl)-1H-imidazole (EMD Millipore) or 15 nM of rapamycin (LC Laboratories). DMSO was used as control. Immediately before injection, a second dose of drug or DMSO was added to the cells. In two additional drug treatment schemes, animals received daily intra-peritoneal (IP) injection of 200μl vehicle (0.2% carboxylmethycellulose/15% Tween-80/5% PEG) with DMSO (used as a solvent to reconstitute both TIE2-TKI and rapamycin), TIE2-TKI (10mg/kg) or rapamycin (2mg/kg). Vascular volume (mm^3) was calculated by 3D Color Doppler measurements performed before and/or at the end of the treatment. Blood gases were measured on an ABL800 FLEX analyzer (Radiometer Medical, Brønshøj, Denmark). Blood cell counts were obtained from an ADVIA 2120 Hematology analyzer (Siemens Diagnostic Solutions, Tarrytown, NY).

Color Doppler Ultrasonography

Vascular volume and blood flow were measured using the Vevo 2100 high-resolution ultrasound system equipped with a MS550D linear array transducer (VisualSonics Inc). The transducer can detect flow in blood vessels with a diameter ≥ 50μm. Animals were anesthetized by isoflurane inhalation, and positioned prone on a movable imaging stage (VisualSonics Inc) heated to 38 °C, with each paw taped to a built-in electrode to monitor respiration and pulse. Analysis of specific channel flow velocity and sound (venous versus arterial) was performed in pulse-waved Doppler mode. A series of images was acquired, via color Doppler mode, at 100μm intervals from the anterior to posterior ends of the lesions. The images were assembled to form a three-dimensional (3D) image stack. To analyze 3D data, the perimeter of the construct was drawn on serial slices 0.5-1 mm apart. The software automatically defined the volume and % vascular volume of the construct by interpolating between the defined regions of interest.

Vessel Density (VD)

For the assessment of VD, ten fields from histological sections of explanted VM lesions were analyzed by counting human CD31+ luminal structures containing red blood cells. Human CD31+ VD was expressed as vessels/mm^2. Vascular area was measured with ImageJ software and 10 fields were analyzed from each explant.

Immunohistochemistry and Immunofluorescence

Paraffin sections of murine VM explants were stained with human-specific anti-CD31 monoclonal antibody (1:50, Dako) or with biotinylated-Ulex Europaeus Agglutinin-I (UEAI) (1:500, Vector Laboratories) to detect human endothelial cells, and with anti-α smooth muscle actin (αSMA) (1:1000, Sigma) to detect smooth muscle/perivascular cells. Incubation with primary antibody was followed by peroxidase-labeled secondary antibody (1:200; Vector Laboratories), and 3,3'-Diaminobenzidine (DAB) staining or by FITC-labeled secondary antibody or Texas Red-labeled Streptavidin for UEA staining (1:200, Vector Laboratories). Nuclei were stained with hematoxylin or 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories).

Microscope Image acquisition

Images were taken with Axiophot II microscope (Zeiss) equipped with AxioCam MRc5 (Zeiss) and using AxioVision Rel.4.6.3 Software. Fluorescence images were taken with Leica TCS SP2 Acousto-Optical Beam Splitter confocal system equipped with DMIRE2 inverted microscope (Diode 405 nm, Argon 488 nm, HeNe 594 nm; Leica Microsystems), Leica Confocal Software Version 2.61, Build 1537. Images were taken at room temperature (about 20 C) and files were exported as 8 bit format.

Immunoblot

Cells were lysed with RIPA buffer (Boston Bioproducts), containing a phosphatase inhibitor cocktail (Roche). Lysates were subjected to SDS-PAGE and transferred to Immibilon-P membrane. Membranes were incubated with antibodies against the following: phospho-mTOR, mTOR, phospho-AKT (Ser473 and Thr308), AKT, phospho-4EBP, 4EBP, phospho-ERK, ERK, phospho-STAT1, STAT1 (all in 1:1000, Cell Signaling Technology), phospho-TIE2 (1:1000, EMD Millipore), TIE2 (1:500, Abcam), Tubulin (1:5000, Sigma-Aldrich). Membranes were incubated with peroxidase-conjugated secondary antibodies (1:5000, Vector Laboratories). Antigen-antibody complexes were visualized using ECL and chemiluminescent sensitive film (Pierce). Band intensity was analyzed with ImageJ software.

Targeted deep-sequencing of TEK coding regions

An Ion AmpliSeq^{TM} Custom DNA Panel (Life Technologies) was used to amplify the 23 coding exons of TEK. The target region of 3,835 base-pairs was covered in 2 primer-pools, with amplicons ranging from 61 to 332 base-pairs. 10ng of DNA (measured on a Qubit 2.0 fluorometer, Life Technologies) was used for library preparation, according to manufacturers' instructions (Ion Ampliseq Library Preparation, Publication MAN0006735, Revision 5.0); multiplex PCR-amplification was followed by partial primer digestion and ligation to two adaptors corresponding to a unique molecular barcode and a universal primer. Each sample therefore yielded two barcoded libraries that together covered the coding region of TEK.

DNA-libraries were purified using AMPure XP magnetic beads (Agencourt) and amplified using a High Fidelity Platinum polymerase and primer mix corresponding to the adaptors. The amplified libraries underwent two rounds of purification using AMPure XP beads. They were subsequently analyzed for quality and concentration on a 2100 Bioanalyzer using the High Sensitivity DNA Kit (both from Agilent Technologies Genomics). Quality was estimated by the presence of peaks at

sizes corresponding to the amplicons, and the region containing these peaks was selected to estimate their molar concentration. Libraries were diluted to 100pM each, and equal volumes of all 18 (2 libraries each from 9 samples, including 5 tissue and 4 blood DNAs) were combined for the remaining steps.

Emulsion PCR was carried out using the Ion OneTouch 200 Template Kit V2 on a OneTouch 2 instrument (Life Technologies) according to manufacturer's protocol (Ion PGMTM Template OT2 200 Kit, Publication MAN0007220, Revision 5.0): DNA strands were hybridized to oligonucleotide-coated beads due to their complementarity to library adapters. Bead-bound DNAs were amplified in individual micro-reactors (air bubbles in an oil-water mixture that contains polymerase and biotinylated primers complementary to library adaptors). Amplification efficiency was estimated in terms of percentage of beads coated with amplicons, using the Ion Sphere Quality Control kit (Life Technologies) on a Qubit fluorometer. Values between 10%-30% were considered acceptable. Amplicon-positive beads were enriched using streptavidin-coated magnetic beads, on an Ion OneTouch ES (Life Technologies). Magnetic beads were detached using a NaOH solution, which was subsequently neutralized.

Prepared templates (bead-bound DNAs) were loaded onto an Ion 318^{TM} Chip v2, a semiconductor chip etched with microwells sized for single beads, and sequenced using the Ion PGM^{TM} Sequencing 200 Kit v2 on an Ion PGM (all from Life Technologies; protocol in Publication MAN0007273, Revision 3.0).

Sequences in the form of FASTQ files, generated by the Torrent Server, were realigned and variants called using NextGENe software (Life Technologies).

SUPPLEMENTARY RESULTS:

Murine VM lesions are similar to patients', enlarge over time and contain non-pulsatile blood flow.

Murine VM lesions were characterized in comparison with infantile hemangioma, a vascular tumor that distinguishes from all the other vascular anomalies for the immunoreactivity to Glucose transporter 1 (GLUT1). Neither patient nor murine VM stained for GLUT1 (**Supplementary Figure S1**).

Hematoxylin and eosin staining of sections from different patient and murine VMs revealed similar blood vessel size and structure, and inflammatory cells were not evident in either patient or murine VM tissue sections (**Supplementary Figure S2**).

We analyzed the murine VM lesions with Doppler ultrasonography and showed that VM blood flow was slow and non-pulsatile (**Supplementary Movie S1-2**).

In addition, 100-800μl blood samples drawn from individual lesions showed characteristics of venous blood, as indicated by high pCO_2 and low pO_2 (**Supplementary Table S1**). Peripheral blood from mice bearing VMs showed increased reticulocyte counts (ranging from 0.6 to 1.7 $x10^6$ reticulocytes/μl in 5/5 lesion bearing mice). Increased reticulocyte counts occurred in association with anemia (5.15-7.55 $x10^6$ RBC/μl in 5/5 lesion-bearing mice with hemoglobin (Hb) values ranging from 9.4-11.6 g/dl in 4/5 lesions) (**Supplementary Table S2**).

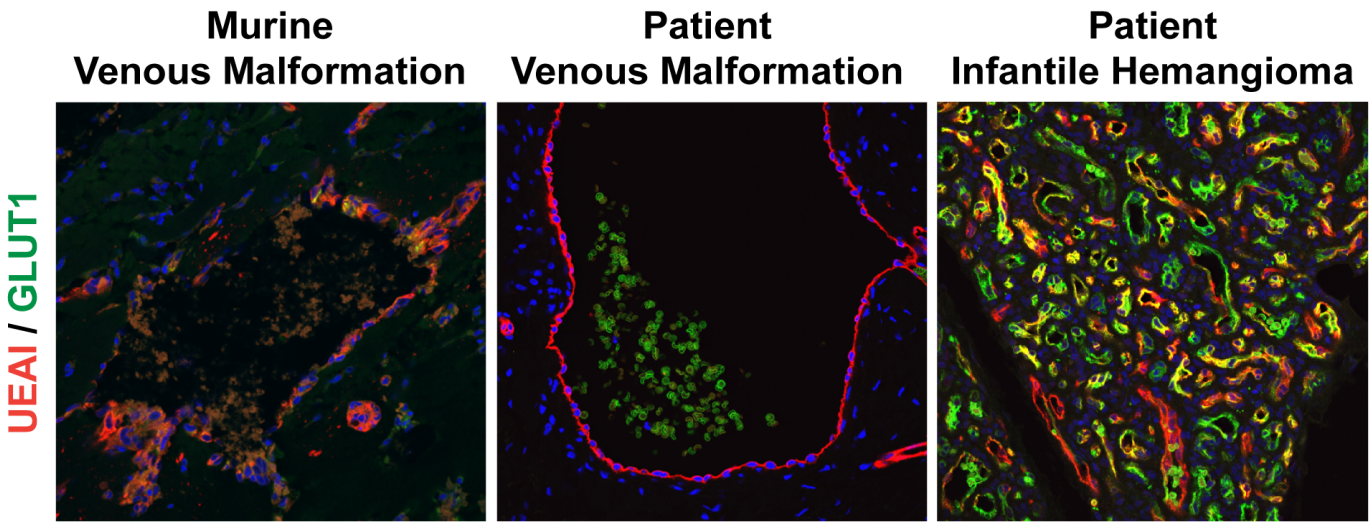
Rapamycin alleviated clinical signs and symptoms in six VM patients.

Clinical parameters are presented in expanded table for each patient, in **Supplementary Table S3**. TIE2 genotyping data are detailed in **Supplementary Table S4**.

REFERENCES:

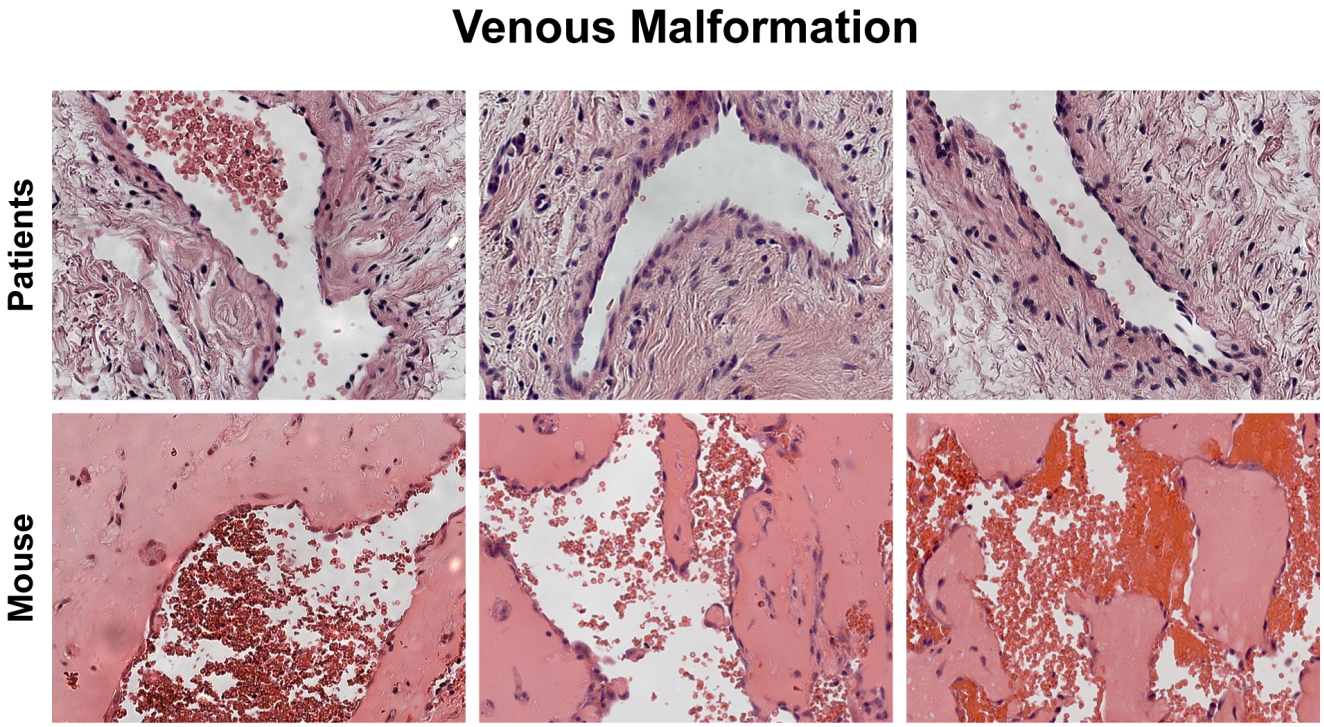
1. Ory, D.S., Neugeboren, B.A., and Mulligan, R.C. 1996. A stable human-derived packaging cell line for production of high titer retrovirus/vesicular stomatitis virus G pseudotypes. *Proceedings of the National Academy of Sciences of the United States of America* 93:11400-11406.
2. Khan, Z.A., Boscolo, E., Picard, A., Psutka, S., Melero-Martin, J.M., Bartch, T.C., Mulliken, J.B., and Bischoff, J. 2008. Multipotential stem cells recapitulate human infantile hemangioma in immunodeficient mice. *J Clin Invest* 118:2592-2599.

SUPPLEMENTARY FIGURES:



Supplementary Figure S1. GLUcose Transporter-1 (GLUT1), specific immunodiagnostic marker for infantile hemangioma, is not expressed in murine and patient venous malformation.

Venous malformation (murine and patient) and patient infantile hemangioma tissue section stained for Ulex europaeus Agglutinin I (UEAI) (red) and GLUcose Transporter-1 (GLUT1) (green). Nuclei are stained with DAPI (blue). Scale bar= 100μm.



Supplementary Figure S2. Immune cells are not evident around venous malformation blood vessels, both in patients and TIE2-L914F dependent murine venous malformations.

Venous malformation (patient and murine) stained with hematoxylin and eosin (H&E).

Movie S1. Pulse-waved Doppler analysis of murine VM- lesion vascular channel. Venous, non-pulsatile blood flow.

Movie S2. Pulse-waved Doppler analysis of murine VM vascular channel on the periphery of the implant. Arterial, pulsatile blood flow.

Supplementary Table S1. Intra-lesional blood gas values.

VM lesion	**pCO_2** (mmHg)	**pO_2** (mmHg)
1	81.4	18.7
2	64.7	26.8
3	68.3	33.6
4	77.2	32.2
mean±SEM	72.9±3.4	27.8±3.0

Supplementary Table S2. Peripheral blood cell counts.

VM lesion bearing-mice	**Reticulocytes (Retic)** x10^6 cells/µl	**Red Blood Cells (RBC)** x10^6 cells/µl	**Hemoglobin (Hb)** g/dL
1	1.70	5.15	9.4
2	0.60	7.41	11.6
3	0.85	6.30	10.1
4	1.44	5.91	10.6
5	0.86	7.55	13.0
mean±SEM	1.09±0.21	6.46±0.45	10.94±0.63
Control mice			
1	**0.16**	**8.75**	**13.5**
2	**0.25**	**8.15**	**12.8**
3	**0.29**	**8.62**	**13.3**
4	**0.31**	**8.58**	**13.2**
mean±SEM	**0.25±0.03**	**8.52±0.13**	**13.2±0.15**
p values	0.0091	0.0058	0.0167
Reference values	0.09 - 0.97	6.36 - 9.42	11.0 - 15.1

Supplementary Table S3: Detailed description of signs and symptoms, and biological and radiological parameters of patients in pilot study.

			Patient 1						Patient 2					Patient 3				
		Reference	At start	3 mos	6 mos	9 mos	12 mos	16 mos	At start	3 mos	6 mos	9 mos	12 mos	At start	3 mos	6 mos	9 mos	12 mos
Rapamycin dosage	Oral intake		na	2 mg/day	2 mg/day	2 mg/day	2 mg/day	1 mg/day	na	2 mg/day	2 mg/day	2-3 mg/day	2 mg/day	na	2 mg/day	2 mg/day	2 mg/day	3 mg/day
Rapamycin level	ng/ml	10 – 15	na	5.4	1.9	4.9	5.6	5.6	na	11.8	11.9	11.5		na	24.9	16.4	3.8	20.5
Evolution Clinical	Personal evaluation of esthetic impairment		+++	+	+	+	+	none	++	+	+	+	+	none	none	none	none	none
	Functional impairment		+++	++	++	+	+	+	+++	+	+	+	none	+++	none	none	+	+
	Pain (VAS)*	0-10	10	8	8	7	6	5	8	1	6	4	1	8	2	2	6	6
	Bleeding		daily	none	none	none	none	none	none	none	none	none	none	none	none	none	none	none
	Infection		none	none	none	none	none	none	none	none	none	none	none	none	none	none	none	none
	Self-perceived QoL	increase	na	30%	20%	20%	30%	50%	na	80%	50%	80%	90%	na	80%	80%	50%	50%
Biological	Hemoglobin (g/dl)	13.0 - 18.0	11.8	12.9	11.6	12.4	12.1	13.3	13.9	13.0	13.1	13.7	15.3	13.7	12.4	12.8	13.9	13.5
	Platelets	150,000 - 350,000	170,000	186,000	154,000	180,000	170,000	206,000	281,000	275,000	187,000	197,000	172,000	175,000	151,000	165,000	172,000	154,000
	D-dimer	<500	12,200	1,565	5,714	3,089	1,699	1,565	12,110	3,264	2,562	1,908	1,183	7,587	2,303	3,022	3,557	1,048
	Fibrinogen (ng/ml)	150 - 450	127	276	276	257	266	245	205	430	372	457	340	319	438	383	254	374
Radiological	MRI		yes	not done	not done	not done	yes	not done	yes	not done	not done	not done	yes	yes	not done	not done	not done	yes
	MRI volume**	cm3 (% reduction)	684	na	na	na	566 (18.1%)	na	1,466	na	na	na	1,291 (11.9%)	19.7	na	na	na	19.6 (0.6%)

			Patient 4					Patient 5						Patient 6	
		Reference	At start	3 mos	6 mos	9 mos	12 mos	at start	3 mos	6 mos	9 mos	12 mos ##	15 mos	at start	3 mos
Rapamycin dosage	Oral intake		na	2 mg/day	2 mg/day	1-2 mg/day	1-2 mg/day	na	2 mg/day	2 mg/day	2 x 2 mg/day	2 x 2 mg/day	2 x 2 mg/day	na	2 mg/day
Rapamycin level	ng/ml	10 – 15	na	10.4	7.3	5.9	8.6	na	3.9	4.6	5.9	6.1	7.4	na	8.1
Evolution Clinical	Personal evaluation of esthetic impairment		none	none	none	none	none	++	+	+	+	+	+	+++	++
	Functional impairment		++	none	none	none	none	+++	++	+	+	+	none	+++	+
	Pain (VAS)*	0-10	8	1	1	0	0	3 - 8#	3	2	2	3	1	10 (daily)	8 (2x/mo)
	Bleeding		none	none	none	none	none	daily	2x/week	1x/mo	none	none	none	none	none
	Infection		none	none	none	none	none	2x/mo	1x/mo	none	none	none	none	none	none
	Self-perceived QoL	increase	na	90%	90%	90%	100%	na	60%	80%	85%	80%	90%	na	30%
Biological	Hemoglobin (g/dl)	13.0 - 18.0	13.1	13.0	13.5	13.2	12.9	11.6	10.9	11.9	12.5	10.6	12.5	14.4	14.7
	Platelets	150,000 - 350,000	272,000	248,000	309,000	245,000	248,000	355,000	276,000	285,000	213,000	233,000	286,000	173,000	192,000
	D-dimer (ng/ml)	<500	663	920	820	560	720	1,685	556	535	481	1,085	800	5,860	2,766
	Fibrinogen (mg/dl)	150 - 450	438	not done	250	269	255	371	376	395	393	421	474	212	235
Radiological	MRI		yes	not done	not done	not done	yes	yes	not done	not done	not done	yes	not done	yes	yes
	MRI volume**	cm3 (% reduction)	14.0	na	na	na	12.3 (12.1%)	362.4	na	na	na	293.7 (18.9%)	na	67.4	61.0 (9.5%)

 # median value (5.5) used in statistical analysis.
*na = not applicable, *p<0.05, Friedman non-parametric repeated measures test; **p<0.05 vs. start, Wilcoxon signed-rank test.*
off Rapamycin for 4 weeks, due to surgical correction of scoliosis

Supplementary Table S4. TIE2 mutations identified in patients by targeted deep-sequencing.

Patient #	Tissue DNA			Blood DNA
	TIE2 mutation	#mutant/#total reads	% mutant reads	TIE2 mutation
1	ND	NA	NA	Not available
2	L914F(c.2740C>T)	316/3088	10.23	ND
3	R1099X(c.3295C>T)	33/984	3.35	ND
4	ND	NA	NA	ND
5	ND	NA	NA	ND
6	L914F(c.2740C>T)	316/3118	10.14	ND

#: number of reads; ND: Not detectable; NA: Not applicable