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Original article

New genetic drivers in hemorrhagic hereditary telangiectasia

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ABSTRACT

Background: Hereditary hemorrhagic telangiectasia (HHT) is a rare vascular disease inherited in an autosomal dominant manner. Disease-causing variants in endoglin (*ENG*) and activin A receptor type II-like 1 (*ACVRL1*) genes are detected in around 90% of the patients; also 2% of patients harbor pathogenic variants at *SMAD4* and *GDF2*. Importantly, the genetic cause of 8% of patients with clinical HHT remains unknown. Here, we present new putative genetic drivers of HHT.

Methods: To identify new HHT genetic drivers, we performed exome sequencing of 19 HHT patients and relatives with unknown HHT genetic etiology. We applied a multistep filtration strategy to catalog deleterious variants and prioritize gene candidates based on their known relevance in endothelial cell biology. Additionally, we performed in vitro validation of one of the identified variants.

Results: We identified variants in the *INHA, HIF1A, JAK2, DNM2, POSTN, ANGPTL4, FOXO1* and *SMAD6* genes as putative drivers in HHT. We have identified the *SMAD6* p.(Glu407Lys) variant in one of the families; this is a loss-of-function variant leading to the activation of the BMP/TGFβ signaling in endothelial cells.

Conclusions: Variants in these genes should be considered for genetic testing in patients with HHT phenotype and negative for *ACVRL1/ENG* mutations.

1. Background

Hereditary hemorrhagic telangiectasia (HHT) or Rendu–Osler–Weber syndrome (ORPHA:774) is a rare autosomal dominant vascular disease characterized by the presence of telangiectases and larger vascular malformations (VM), mainly in pulmonary, hepatic or cerebral vasculature [1]. Telangiectasis is the main characteristic lesion in HHT and consists in dilated postcapillary venules directly connected to dilated arterioles bypassing the normal capillary bed [2]. Nasal or gastrointestinal mucosae telangiectases are more prone to hemorrhage, causing epistaxis, gastrointestinal bleeding and anemia, being these the clinical hallmarks of the disease [3].

HHT can be diagnosed either clinically using the Curaçao criteria (recurrent epistaxis, mucocutaneous telangiectasia, visceral VMs and family history), and/or through molecular gene test [4,5]. Loss-of-function variants in the *ENG* (endoglin) and *ACVRL1* (activin A

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receptor type II-like 1) genes are detected in approximately 90% of patients submitted to molecular diagnosis for clinical suspicion of HHT. Variants in the *ENG* gene cause type 1 HHT (HHT1; OMIM:187300) characterized by pulmonary arteriovenous malformations (AVMs) and brain VMs whereas *ACVRL1* variants cause type 2 HHT (HHT2; OMIM:600376) where hepatic VMs are more prevalent [6,7].

Endoglin is an auxiliary co-receptor at the endothelial cell (EC) surface that promotes BMP9/10 signaling through the activin receptor-like kinase 1 receptor (ALK1; encoded by *ACVRL1*). Together, they contribute to the activation of endothelial BMP/SMAD signaling having an essential role in angiogenesis [8]. Other variants affecting genes related to this canonical pathway such as *SMAD4* or *GDF2* (BMP9) genes have also been described and account for less than 2% of HHT patients [9,10]. Additionally, some other diseases that also result in AVMs may overlap with HHT clinical features such as CM-AVM type 1 and 2, caused by *RASA1* and *EPHB4* mutations respectively, and may also be tested in case of a clinical suspicion of HHT but negative genetic testing [11,12]. Importantly, the genetic cause of 8% of the patients with clinical HHT, remains unknown [5,6]. These, amongst other unknown HHT fundamentals, have hampered the discovery of curative treatments. Also, patients with undiagnosed rare diseases can only receive symptomatic therapies and therefore, an accurate diagnosis can result in better management of the disease, identification of potential therapeutics and avoid unnecessary treatments that may have severe side effects. Moreover, knowing the causative variant and the mode of inheritance informs patients about the risk of passing the disease to future generations and helps evaluate alternatative family planning options [13].

Our group is a translational team focused on uncovering HHT mechanistic processes with a bench-to-bedside approach [14–16]. We analyzed data from the largest cohort of HHT patients with non-canonical HHT variants ever submitted for whole exome sequencing and propose new genetic drivers for these individuals with an HHT-like phenotype but unknown genetic background.

2. Material and methods

2.1. Study participants and clinical data

We selected patients attended in a referral HHT Unit in a university hospital that caters for adult patients from all over Catalonia (Spain), which has about 7.5 million inhabitants. All patients from our database with a definite HHT diagnosis according to the Curaçao Criteria (meet $\geq$3 criteria) and a negative genetic test were included. Genetic diagnosis is performed by a Next Generation Sequencing (NGS) panel that includes 9 genes (*ACVRL1, BMP10, BMPR1A, BMPR2, ENG, GDF2, RASA1, SMAD1, SMAD4*) previously related to HHT or other vascular diseases with similar phenotypes. The detection of copy number variations (CNVs) was carried out using a normalized coverage comparison approach and when suspected, confirmed or discarded by Multiplex Ligation-dependent Probe Amplification (MLPA).

According to Helsinki Declaration, all participants provided their signed informed consent for participation in the study following local Ethics Committee requirements. Personal and clinical data collected for the study are in line with the Spanish Data Protection Act (Ley Orgánica 3/2018 de 5 de diciembre de Protección de Datos Personales). Accordingly, all individual-level data was de-identified. The study was approved by the Clinical Research Ethics Committee of the Hospital Universitari de Bellvitge (approval number PR203/21).

Patients' clinical data was compiled from the HHT Unit database. Baseline demographic characteristics, comorbidities, blood test values, and Epistaxis Severity Score (ESS) were collected. ESS is an online tool that quantifies epistaxis severity considering different parameters during last three months [17]. A meticulous physical examination was performed to identify telangiectases present in skin and oral/nasal mucosae. Screening for pulmonary AVMs, was performed with contrast transthoracic echocardiography (TTE) and, depending on the degree of a right-to-left shunt, a thoracic computed tomography (CT) angiography was performed [7,18]. Hepatic and abdominal VMs were screened using abdominal CT angiography. Brain VMs were studied with cerebral CT angiography or brain magnetic resonance imaging (MRI) if neurological symptoms or family history were detected [7,19]. GI endoscopic study was performed in patients with disproportionate anemia to the amount and severity of epistaxis [5,20].

An interview was scheduled to double check clinical data from the database, extract patients' blood and build a pedigree of the family. Pedigrees were built using TreeStudio 2.0 software. For cosegregation studies, we aimed to select at least one affected and one healthy relative when available and visited them in the outpatient clinics to collect the same data than from the probands. Blood samples were anonymized and sent to our Biobank for storage and management.

2.2. Reagents

hTERT neonatal dermal microvascular endothelial cells (hTERT-DMECs; #CRL-4060) were obtained from ATCC. EGM-2 (#C-22111) medium was obtained from Promocell. BMP9 (#120-07) is from Preprotech. Antibodies: anti-SMAD6 (NB100-56440, NovusBio), anti-ID1 (#SC-488, Santa Cruz), anti-vinculin (#V9131, Sigma Chemical), anti-SGK1 (#012103, Cell Signalling). Secondary antibodies anti-rabbit and anti-mouse (Amersham Pharma-cia Biotech, #NA934 and #NXA931, respectively). CS2-HA-SMAD6 plasmid (Addgene, #14,962).

2.3. DNA extraction

DNA was extracted by Biobank HUB-ICO-IDIBELL using the Maxwell® 16 Blood DNA Purification Kit (Promega Corporation, Madison, USA) from peripheral blood mononuclear cells (PBMCs), according to the manufacturer's recommendations. Genomic DNA was then quantified using an Implen 0810 spectrophotometer (Implen GmbH, München, Germany) and sent for analysis.

2.4. Exome sequencing and data analysis

Exome sequencing was performed on DNA extracted from participants PBMCs. DNA library was prepared with KAPA HyperPrep library kit (Roche, Pleasanton, CA, US), hybridized with a SeqCap EZ MedExome capture kit (Roche) and sequenced on a NS500 (Illumina Inc. San Diego, CA, USA) to produce 2 $\times$ 150 bp paired-end reads. Mean average depth of on-target coverage in the sequenced exome was 60$\times$. Single nucleotide variants (SNVs) and small insertions and deletions were identified after aligning to the human reference genome (GRCh37) using BWA (v.0.7.12). Variant calling was done using GATK callers (HaplotypeCaller and UnifiedGenotyper) as per protocol recommendations [21].

To prioritize candidate variants, we used a multistep filtration approach. First, variants were filtered by location, including exons or their flanking intronic regions. Then, we selected variants with a minor allele frequency (MAF) in the general population (gnomAD database) less than 1/1000 or unknown. We discarded those already categorized as benign or likely benign variants by ClinVar [22]. Next, we performed a sequencing quality assessment by reviewing BAM files in IGV software. Importantly, we selected heterozygote variants only present in the proband and affected relatives, and not in the healthy ones (co-segregation). Afterwards, we did a pathogenicity analysis for each variant applying the American College of Medical Genetics and Genomic (ACMG) guidelines [23] by using Varsome, which additionally allows to perform multiple in silico pathogenicity analyses simultaneously [24]. With Varsome information, we discarded those variants identified as benign or likely benign and those of uncertain significance without any pathogenic consideration in the four evaluated Meta Scores (MetaLR, MetaSVM, Meta RNN, and REVEL) and subsequently, we cataloged the remainders as potentially deleterious variants.

To test the biological relevance in a vascular disease environment of the genes harboring the identified potentially deleterious variants, we used the Human Gene Connectome server (HGCS) [25,26]. The HGCS enables researchers to prioritize any list of genes by their biological proximity to defined core genes. The HGC is the set of all biologically plausible routes, distances, and degrees of separation between all pairs of human genes. The server provides a numeric distance between two genes and a p-value specifying the significance of the proximity of the gene of interest to a core gene; this allows ranking a set of genes depending on the proximity to the core genes. With this, we ranked the genes harboring potentially deleterious variants by biological proximity to HHT driver genes (*ACVRL1* and *ENG*). Finally, we performed a one-by-one gene search in Gene Ontology resource [27,28], Panther Classification System [29], Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database [30], Human Phenotype Onthology (HPO) [31] and Mastermind [32] gathering all the codes and relevant published papers related to each gene to apprehend all possible information about its biological function, participation in biological processes, role in different signaling pathways and correlation to human symptoms and diseases.

2.5. Mutagenesis

PCR-based mutagenesis was applied to create single-point (c.1219G > A; p.E407K) mutation in CS2-HA-SMAD6 plasmid purchased from Addgene (#14962). Primers were designed using QuikChange Primer Design Agilent Tool (5′-CCGCGGCAAGCACCCCATCTTCG-3′ and 5′-GGGTGCTTGCCGCGGTTGTAGGC-3′). After temperature cycling, the parental template was digested by DpnI (1.5 h at 37 °C), and the mutated plasmid was transformed into competent Dh5α cells. Plasmid was isolated from five selected colonies and sent for DNA Sanger sequencing to verify the presence of the mutation.

2.6. Generation of SMAD6 knock-out hTERT-HDMECs by CRISPR/Cas9

gRNAs targeting two different regions of *SMAD6* exon 1 (5′-CACCGTATGTTCAGGTCCAAACGCT-3′ and 3′-AAACAGCGTTTGGACCTGAACATAC-5′) and (5′-CACCGCGACTTTGGCGAAGTCGTG-3′ and 3′-AAACCACGACTTCGCCAAAGTCGC-5′) were inserted in the pSpCas9(BB)-2A-GFP (PX458) plasmid (Addgene #48138). hTERT-HDMECs (3×10^5 cells) were transfected with 2.5 μg of SMAD6-gRNA-PX458 plasmid using lipofectamine 3000 (ThermoFisher #L3000015) following manufacturer instructions. Forty-eight hours after transfection, GFP-positive cells were FAC-sorted (FACSAria II; BD Biosciences) and plated individually in 100 μl of EGM2 medium in 96-well plates. Individual clones were visible after two weeks and then expanded.

2.7. Cell transfection

3×10^5 cells were seeded onto 0.5% gelatin-coated wells (6-well format) and cultured overnight at 37 °C in 5% CO_2 atmosphere in EGM-2 medium without antibiotics and heparin. The next day, cells were transfected with CS2-HA-SMAD6^{WT} or CS2-HA-SMAD6^{E407K} using Lipofectamine® 3000 (Invitrogen, L3000008) following the manufacturer's instructions. After 8 h incubation with transfection mix, cells were refreshed with complete EGM-2 medium and cultured for additional 48 h.

2.8. Western blot

Cells were collected and lysed in RIPA lysis buffer (PBS pH 7.4, SDS 0.1%, NP-40 1%, sodium deoxycholate 0.5%) with protease and phosphatase inhibitors. SDS/PAGE was used to separate proteins, which were then electrophoretically transferred to Immobilon-P membranes (Millipore, #IPVH00010). Membranes were blocked in TBS (10 mM Tris/HCl, 150 mM NaCl, pH 7.4) with 5% of nonfat dry milk. Blots were incubated with 1:1000 primary antibody for all proteins except for ID1, that was at 1/500, in TBS 1% nonfat dry milk at 4 °C overnight. After washing in TBS 0.1% Triton X-100, blots were incubated with secondary horseradish peroxidase linked antibodies, in TBS 1% nonfat milk. Blots were washed in TBS 0.1% Triton X-100, and then, ECL Prime (Amersham Pharmacia Biotech; Cat. Num. #RPN2236) was added and measured with the ChemiDoc™ MP imaging system. Volumetric analysis was performed using IMAGE LAB from Bio-Rad (Hercules, CA, USA).

3. Results

3.1. Patients' selection and clinical characteristics

In our clinical Unit we currently manage 430 HHT patients. From them, 18 patients have an HHT diagnosis according to the Curaçao Criteria (meet ≥3 criteria) and a negative genetic test for known HHT genetic drivers (*ACVRL1, BMP10, BMPR1A, BMPR2, ENG, GDF2, RASA1, SMAD1, SMAD4*). Additionally to these 18 patients, six unaffected relatives were recruited. With this, 13 individuals (including two monozygotic twins) were members of seven different families and five were unique probands (singletons). Singletons were not considered for analysis due to the impossibility to perform cosegregation; thus, a total of 19 exomes were analyzed. A flow chart of participants selection is available in Suppl. Mat. 1. Clinical and demographic data of the participants are summarized in Table 1 and clinical representative images of patients are shown in Fig. 1. A pedigree of each family is available in Suppl. Mat. 2.

3.2. Identification of new variants in HHT-like families

To prioritize candidate variants from WES analyses, we used the aforementioned multistep filtration approach (Fig. 2(A)). After this, we cataloged 26 variants as potentially deleterious (Suppl. Mat. 3); among them, 24 were missense, one was a frameshift insertion and one an alternative splicing. Then, we evaluated the biological distance of candidate genes to HHT driver genes through HGCS; a figure depicting biological proximity of the selected genes to the HHT core genes (*ACVRL1* and *ENG*) by the use of HGCS is available in Fig. 2(B). Variants in the *INHA, HIF1A, JAK2, DNM2, POSTN, ANGLPT4, FOXO1* and *SMAD6* genes were considered as putative drivers of HHT-like phenotype (Table 2; Suppl. Mat. 4.).

For **family A** we considered *INHA* p.(Pro308Ser) variant as driver of the disease. *INHA* encodes for the alpha subunit of inhibin, a distinct and unique member of the TGFβ family that is an endocrine hormone. *INHA* variants have been associated with ovarian insufficiency and preeclampsia, but it also has a role in hypoxia-induced tumor growth and vascular permeability downstream endoglin and ALK1, and it has been implicated in regulating metastasis and vascular functions through paracrine signaling, in ECs [33,34]. Our genetic studies in **family B** have identified a germline missense variant p.(Met60Ile) in the *HIF1A* gene. *HIF1A* is a master transcriptional regulator of the adaptive response to hypoxia and induces the transcription of genes activating angiogenesis, VEGF production and ECs proliferation and migration. Interestingly, it has been recently reported that its expression is downregulated in HHT patients compared to healthy controls [35]. For **family C** we have found a germline variant p.(Tyr62Phe) in *JAK2* gene. *JAK2* encodes for a non-receptor tyrosine kinase that activates SMAD-related signal transduction and PI3K signaling in the vasculature; also, it is associated to smooth muscle cell proliferation [27,28]. *JAK2* somatic gain-of-function variants have been related to different diseases such as Budd-Chiari syndrome, essential thrombocythemia, polycythemia vera and primary myelofibrosis [36,37]. In **family E** we identified a missense variant in *DNM2* p.(Ile365Val). The *DNM2* gene encodes for a microtubule-associated protein that binds to the phosphatidylinositol-4,

Table 1
Patients demographic and clinical basic data. M, male; F, female; FH, family history; A, atypical; F, finger; L, lip; T, tongue; N, nose; VMs, vascular malformations; GI, gastrointestinal; Lu, lungs; Li, liver; Oth, other localizations; n/a, not assessed.

Family ID	Patient ID	Kinship	Sex	Age	Curaçao criteria									
					FH	Epistaxis	Telangiectasia					Visceral VMs			
							A	F	L	T	N	GI	Lu	Li	Oth
A	1	Proband	F	78	x	x		x	x	x	x	n/a		x	x
	2	Twin	F	78	x	x		x	x	x	x	x		x	x
	3	Son	M	51	x	x		x	x	x	x	x			x
	Niece		F	47	Unaffected										
B	4	Proband	M	72		x					x	x		x	
	Son		M	43	Unaffected										
C	5	Proband	F	23		x	x		x			x		x	
	6	Brother	M	19	x	x		x			x				
	Sister		F	14	Unaffected										
D	7	Proband	M	60	x	x	x		x		x	x		x	
	Son		M	18	Unaffected										
E	8	Proband	F	44	x	x					x			x	x
	9	Brother	M	59	x	x		x	x		x	n/a			
	Nephew		M	19	Unaffected										
F	10	Proband	F	63	x	x		x	x	x	x	n/a	x		
	11	Daughter	F	36	x			x	x			n/a		x	
G	12	Proband	F	30	x		x					x		x	
	13	Father	M	55			x					x			
	Sister		F	20	Unaffected										

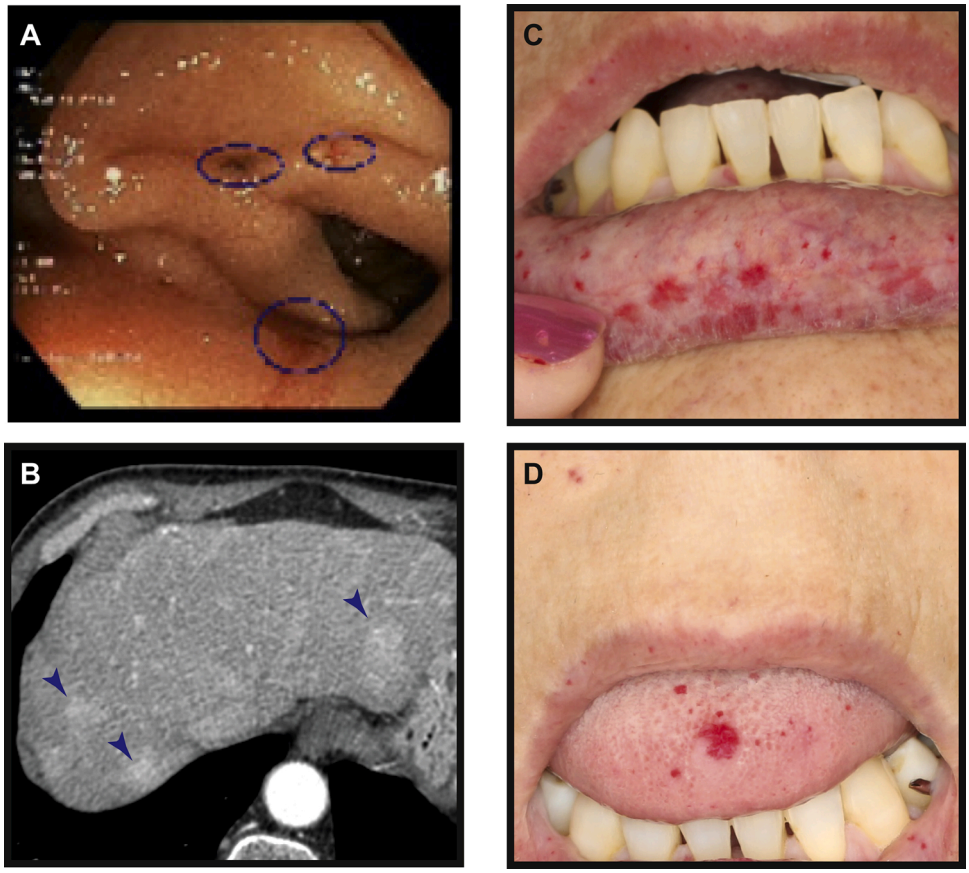


Fig. 1. Photographs and radiologic imaging of Patients 4 and 10 with HHT phenotype.
Patient 4. Gastrointestinal telangiectases identified through endoscopic capsule performed because of anemia disproportionate to epistaxis (A) and liver arteriovenous malformations image from contrast enhanced computerized tomography (B). Patient 10. Lips (C) and tongue (D) typical telangiectases.

5-bisphosphate [PI(4,5)P_2] having an important role in vesicular trafficking [38]. Previously, loss-of-function missense variants in *DNM2* have been linked to autosomal dominant centronuclear myopathy and Charcot-Marie-Tooth disease type B [39]. Also, it has been related to endothelial dysfunction in hypoxic pulmonary hypertension in silico [40]. Interestingly, for **family G** we identified a missense variant p.(Glu407Lys) in the *SMAD6* gene which encodes for an inhibitor SMAD (I-SMAD), a key regulator of the ALK1/Endoglin/SMAD4 signaling hub. In a preclinical mouse model, the loss of *SMAD6* leads to blood vessel hemorrhage and disrupted EC junctions [41]. Also, loss-of-function

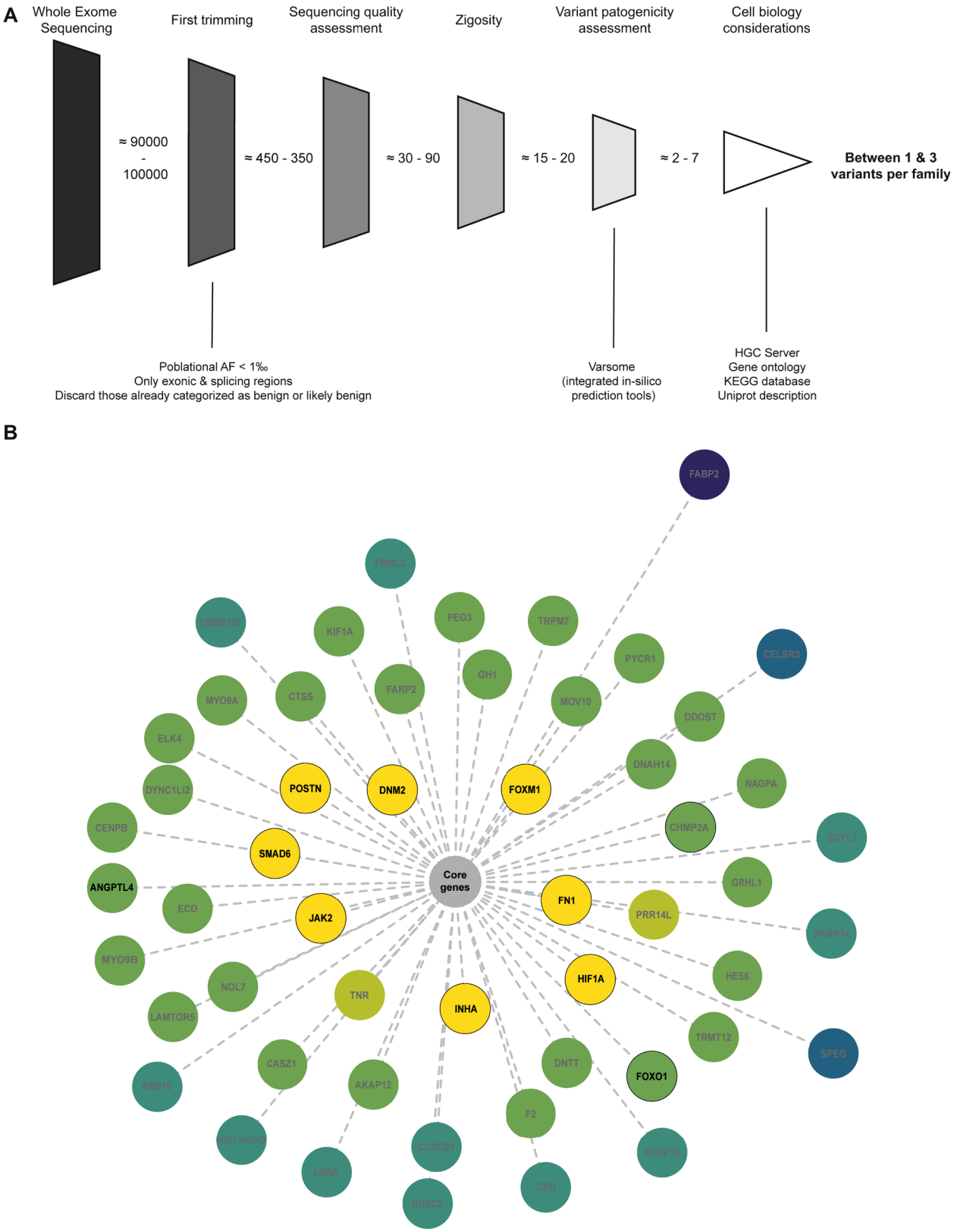


Fig. 2. Exome sequencing analysis and candidate variants prioritization.
(A) Variant trimming strategy used for the whole exome sequencing analysis. Beginning from raw data (around 90,000 variants per patient) different filters related to variant frequency in general population, quality of the reads, zygosity, variant pathogenicity assessment through Varsome, calculation of the biological distance to *ENG* and *ACVRL1* genes using the Human Gene Connectome Server and detection of endothelial biological relevance through Gene Onthology and KEGG terms. (B) Graphical representation of the biological distance between core genes and genes harboring potentially deleterious variants identified in the HHT like families. Core genes are *ACVRL1* and *ENG*. Those genes with a distance *p*-value <0.05 have a black stroke and those selected as putative drivers of HHT like phenotypes are written in bold Black.

Table 2
List of selected variants in affected families. VUS, variant of uncertain significance; LP, likely pathogenic; P, pathogenic.

Family	Gene	RefSeq	Nucleotide change	Aminoacid change	Variant type	InterVar
A	*INHA*	NM_002191.3	c.922C > T	p.(Pro308Ser)	Missense	VUS
B	*HIF1A*	NM_001530.3	c.180G > T	p.(Met60Ile)	Missense	VUS / LP
C	*JAK2*	NM_001322194.1	c.185A > T	p.(Tyr62Phe)	Missense	VUS
E	*DNM2*	NM_001005360.2	c.1093A > G	p.(Ile365Val)	Missense	P
F	*POSTN*	NM_001135934.2	c.1090G > A	p.(Val364Ile)	Missense	VUS
	FOXO1	NM_002015.3	c.1340C > T	p.(Ser447Leu)	Missense	VUS
	ANGPTL4	NM_001039667.3	c.860C > T	p.(Pro287Leu)	Missense	VUS
G	*SMAD6*	NM_005585.4	c.1219G > A	p.(Glu407Lys)	Missense	VUS

germline missense variants in *SMAD6* MH2 domain are related with familial bicuspid aortic valve [42]. In **family F**, we identified 3 missense variants of uncertain significance (VUS) in three different genes with biological relevance in vascular homeostasis: *POSTN* p.(Val364Ile), *FOXO1* p.(Ser447Leu), and *ANGTPL4* p.(Pro287Leu). *POSTN* gene encodes for Periostin, a TGFβ-inducible secreted protein. *POSTN* expression is increased in pulmonary aortic smooth muscle cells in response to hypoxia [43], and it induces angiogenesis in rheumatic valve degeneration [44]. FOXO1 is a transcription factor that is an essential regulator of vascular growth. FOXO1 translocation to the nucleus is inhibited by PI3K signaling activation regulating metabolic and proliferative activities in ECs [45]. *ANGTPL4* has not been considered by the HGCS as of close proximity to HHT driver genes but it codes for Angiopoietin Like 4, a well-known angiogenic factor that functions as a 'gatekeeper' regulating vascular integrity in a context-dependent manner [46]. Although *ANGPTL4* has not been directly linked to any disease, specific variants have been proposed to be a risk factors for brain arteriovenous malformations [47]. Moreover, *HIF1A* is directly linked to *ANGLPT4* as it promotes its upregulation in hypoxic retinal Müller cells, promoting vascular permeability [48]. The key relevance of this gene in vascular biology suggests that might have a role on HHT-like phenotype.

Regarding **family D**, a missense VUS was identified in *F2*, a gene that encodes the prothrombin protein and plays an important role in thrombosis and hemostasis, being related to both thrombotic and bleeding disorders. A gain-of-function variant causes the most common inherited thrombophilia together with factor V Leiden and therefore, is related to increased risk of venous thromboembolism [49]. On the other hand, loss-of-function variants in *F2* cause congenital factor II deficiency disease, that leads to early in life severe bleedings and epistaxis [31]. Although this patient has a clinical history of anemia related to his gastrointestinal bleeding and epistaxis, the lack of alterations in coagulation coupled with the presence of mucocutaneous telangiectases, suggests that variant in *F2* is not the genetic driver for this patient HHT-like phenotype.

3.3. *SMAD6 is a candidate for HHT pathogenesis*

We then assessed the impact of one of our candidates on endothelial cell biology. Given the relevance of the BMP/SMAD signaling in the disease, we analyzed the *SMAD6* p.(Glu407Lys) variant identified in family G. *SMAD6* is an inhibitory SMAD that downregulates BMP/SMAD signaling upon BMP9 stimulation [50,51]. Specifically, this mutation is located in the MH2 domain (Fig. 3(A)) which is critical for the negative regulation of BMP/TGF-β signaling serving as a docking site for target proteins, such as type I TGF-β receptors and R-SMADS [51]. To analyze the impact of *SMAD6* p.Glu407Lys (E407K) variant on BMP/SMAD signaling, we first generated *SMAD6* knock-out (KO) immortalized dermal microvascular ECs (hTERT-DMECs) by CRISPR/Cas9. These cells have a deletion leading to a premature stop codon (NM_005585.4: c.50del; p.Arg17Leufs*47). We analyzed the expression of SGK1 and the ID1 transcription factor as read-outs for BMP/SMAD signaling [50,52] in $SMAD^{KO}$ ECs with exogenous expression of either $SMAD6^{WT}$ or $SMAD6^{E407K}$, and upon BMP9 stimulation (10 ng/mL) during 0, 2, and 6 h in growth factor-depleted ECs (Fig. 3(B) and (C)). With this, we found that endogenous *SMAD6* is expressed after 6 h of BMP9 stimulation and demonstrated that $SMAD6^{KO}$ ECs showed enhanced expression of SGK1 and ID1, validating the role of SMAD6 as an inhibitory SMAD (Fig. 3(B) and (C)). Importantly, exogenous expression of $SMAD6^{WT}$ partially rescues the overactivation of BMP/SMAD signaling in $SMAD6^{KO}$ ECs; however, the $SMAD6^{E407K}$ variant is unable to downregulate the signaling in these cells, implying the loss of function of the *SMAD6* inhibitory activity in BMP/SMAD signaling.

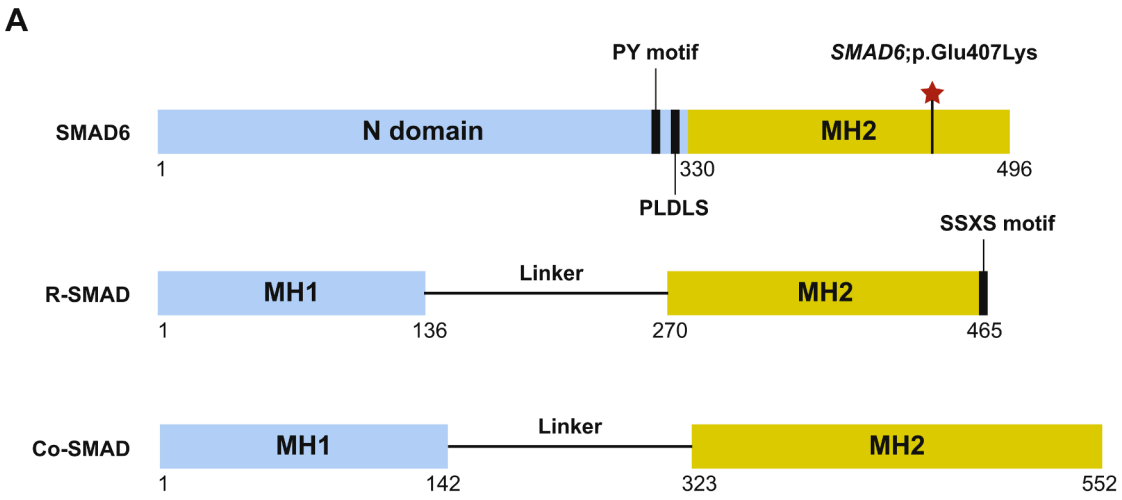
4. Discussion

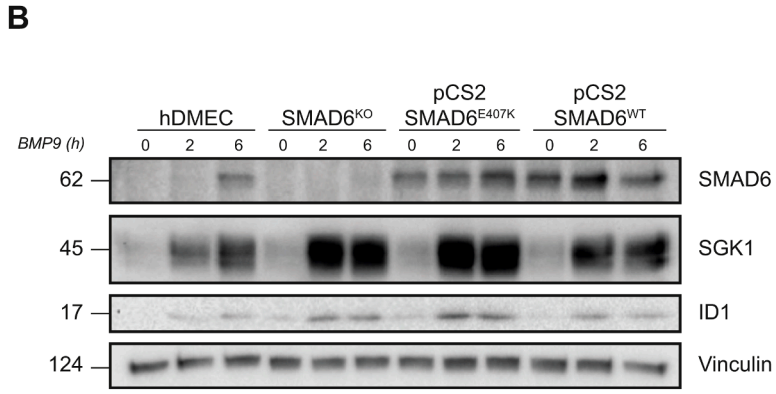
To the best of our knowledge this is the largest published record of WES analyses in genetically orphan HHT families. WES sequencing has been increasingly used to identify new pathogenic variants and new genes related to different conditions, mainly in rare diseases [13,53,54]. Since the main HHT genetic landscape (*ENG* and *ACVRL1* variants) was first described in the 90s [55,56], efforts have been done to uncover additional genes related to the phenotype, and new genetic drivers have been added to HHT molecular diagnose tools in the subsequent years (*SMAD4, GDF2, RASA1, EPBH4*, etc.) [9–12]. However, there are still at least 8% of families that fulfill the clinical criteria but do not exhibit any potentially known pathogenic variant in any of these mentioned genes. Here, we present eight potentially pathogenic germline variants in genes with a key role in angiogenesis and EC biology.

A remarkable observation from our data is that there is no common pathogenic germline variant driver amongst the seven families. This suggests that these mutations are rare and quite likely explains why it is taking so long to identify the genetic cause of the 8% orphan cases in HHT. Nevertheless, as genetics diagnose is increasingly wider available, more patients are analyzed and, in consequence, more clinical phenotypes are related to genes that have only been described in one patient or a single family [57,58]. Also, in silico prediction tools of pathogenicity and biological relevance are continuously being updated and refined. This has allowed accurate and precise prioritization of variants in this work to identify driver variants [59]. Additionally, the strength of our results resides in that the variants we have identified do not come from singletons, but from families with two, or three affected relatives and that we provide functional assays on one of these variants as a proof of the validity of the used strategy.

When looking into the HHT genetic landscape, there is always a gap explaining heterogeneous phenotypes even with same mutated genes and within same families, despite the germinal nature of the disease. Nonetheless, different groups suggested various hypothesis [60] that range from the loss of heterozygosity [61] to the presence of modulating variants (deleterious variants on top of the pathogenic ones in *ENG* or *ACVRL1*) in genes not directly related to HHT but to hemostasis and hemolysis processes [62]. In line with this, there is some phenotypic heterogeneity among affected patients from the same family and we have not observed specific traits related to the new variants identified.

SMAD6 encodes an intracellular inhibitor of the BMP/SMAD signaling hub and it is required for EC flow-mediated responses to maintain vascular homeostasis [63]. Recently, it has been demonstrated that SMAD6 maintains a balance of PI3K signaling through the negative modulation of ALK1 to regulate EC contractility and vessel integrity





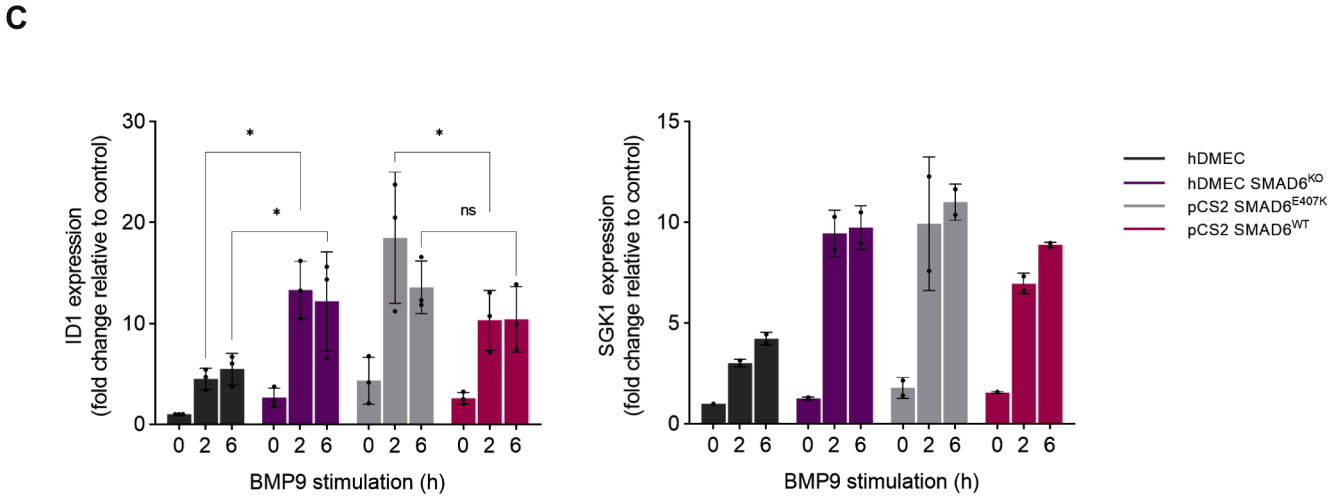


Fig. 3. SMAD6 p.(Glu407Lys) functional studies as a proof of concept of strategy validity.
(A) Schematic representation of the structure of SMAD6 (I-SMAD) and the position of SMAD6 p.(Glu407Lys) variant in the MH2 domain. The amino-terminal regions (N domains) of I-SMADs diverge from the MH1 domains and linker regions of R-SMADs and common-partner SMADs (co-SMADs), but the carboxy-terminal MH2 domains are highly conserved amongst them. I-SMADs inhibit TGFβ family signaling through multiple mechanisms, among which interactions with activated type I receptors and activated R-SMADs are crucial for the inhibition of SMAD-mediated signaling. The MH2 domains are required for interactions with activated type I receptors and R-SMADs. PY, Pro-Tyr; PLDLS, Pro-Leu-Asp-Leu-Ser; Ser-Ser-X-Ser (B) Western blot assessing SGK1 and ID1 expression as read-outs for TGFβ/SMAD signaling in control HDMECs, *SMAD6*^{KO} HDMECs, and *SMAD6*^{WT}- and *SMAD6*^{E407K}-transfected *SMAD6*^{KO} HDMECs. Growth factor-depleted cells were stimulated with 10 ng/mL of BMP9 for the indicated times. (C) Western blot quantification. Bars represent the mean and SEM of ID1 and SGK1 expression over vinculin. $n = 3$ independent experiments for ID1 expression; $n = 2$ independent experiments for SGK1 expression. ns $p > 0.05$; * $p < 0.05$; Uncorrected Fisher's test for multiple comparisons upon one-way ANOVA.

[64]. Indeed, our finding further highlights the importance of the balance of the TGFβ/SMAD signaling pathway to maintain homeostasis in ECs and that its dysregulation (too little or too much) is a trigger of HHT pathogenesis. Also, given our observation of the impact of *SMAD6* loss-of-function on ID1 and SGK1 expression downstream, we analyzed our WES data for either ID1 or SGK1 variants prior to our filtering strategy to find any putative modifier variant; however, we did not find any. *SMAD6*-deficiency caused by germline loss-of-function mutations has been previously associated with three distinct human congenital conditions [65]. Interestingly, identical nucleotide changes have been described in patients with different pathological phenotypes; indeed, this is the first time that *SMAD6* has been related to HHT.

Another interesting result is that more than half of the new proposed genes (*INHA, HIF1A, DNM2, POSTN, ANGPTL4*) are related to EC hypoxia responsiveness. Hypoxia has been suggested as a possible non-genetic second hit for HHT VMs development, but very little has been published as of today [60,66,67]. Here we propose that, in addition to the role that environmental hypoxia exerts on VMs development, dysregulation on the intrinsic responsiveness of ECs to hypoxia through pathogenic variants contributes to the pathogenesis of these diseases. Therefore, there is a need to better understand the role of hypoxia in HHT pathophysiology.

The present study has some strengths and limitations that should be mentioned. On the one hand, we have only focused on coding regions of the genome and therefore may have missed potentially deleterious deep intronic variants. Indeed, there is one patient for whom we have not identified any possible pathogenic variant related to angiogenesis. On the other hand, there is a lack of functional studies for all the prioritized variants that support in silico assessed variant pathogenicity in an endothelial context. Yet, the strategy for variant prioritization is accurate and robust, supported by cosegregation studies and state-of-the-art updated in silico tools. In addition, the identification of novel HHT driven variants is critical for the generation of preclinical models towards the development of new therapeutic strategies.

In conclusion, we provide eight new candidate drivers for HHT-like disease that should be tested in patients with an HHT phenotype and no genetic diagnosis after molecular testing for the usual suspects. SMAD6 p.(Glu407Lys) in vitro assays support its pathogenic role in HHT phenotype and the validity of our filtration strategy. Additionally, we bring into focus hypoxia and its related genes as important players in vascular malformation development in HHT scenario.

Ethics approval and consent to participate

The research has been conducted in accordance with Helsinki Declaration. All participants provided their signed informed consent for participation in the study following local Ethics Committee requirements. Personal and clinical data that were collected for the study are in line with the Spanish Data Protection Act (Ley Orgánica 3/2018 de 5 de diciembre de Protección de Datos Personales). Accordingly, all individual-level data was de-identified. The study was approved by the Clinical Research Ethics Committee of the Hospital Universitari de Bellvitge (approval number PR203/21).

Consent for publication

All patients' images were taken after a specific and separate consent was collected, and publication permission was specifically asked.

Availability of data and materials

The raw datasets generated and/or analyzed during the current study are not publicly available due to personal data restrictions but are available from the corresponding authors on request. This excludes any individual personal/clinical data of the individuals, which would endanger their anonymity.

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CRediT authorship contribution statement

Pau Cerdà: Data curation, Formal analysis, Resources, Writing – original draft. **Sandra D. Castillo:** Conceptualization, Data curation, Formal analysis, Resources, Writing – original draft. **Cinthia Aguilera:** Formal analysis. **Adriana Iriarte:** Data curation, Writing – original draft. **José Luis Rocamora:** Data curation, Formal analysis. **Ane M. Larrinaga:** Formal analysis. **Francesc Viñals:** Conceptualization, Writing – review & editing. **Mariona Graupera:** Conceptualization, Formal analysis, Writing – original draft, Resources, Writing – review & editing. **Antoni Riera-Mestre:** Conceptualization, Formal analysis, Writing – original draft, Resources, Writing – review & editing.

Declaration of Competing Interest

Authors declare that they have no conflict of interest.

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