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Angiopoietin-1 haploinsufficiency affects the endothelial barrier and causes hereditary angioedema

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Summary

Background: Different mutations of the angiopoietin-1 gene (*ANGPT1*) have been associated with the occurrence of hereditary angioedema (HAE).

Objective: The purpose of the study is to clarify whether the *ANGPT1* A119S variant plays its role via haploinsufficiency or a dominant negative effect.

Methods: The ability of *ANGPT1* A119S variant to affect the endothelial barrier function was assessed by immunocytochemistry. Inter-endothelial gap formation molecules primarily responsible for cell-cell adhesions of HUVECs, vascular endothelial (VE)-cadherin and β-catenin, and reorganization of the F-actin cytoskeletal were evaluated.

Results: In in vitro conditions mimicking the heterozygous state, the p.A119S variant significantly reduced the capability to bind its natural receptor (80.7% of normal), less than the homozygous condition (59.1%). After stimulation of VEGF or bradykinin, the addiction to equimolar amounts of wtANGPT1 and ANGPT1 p.A119S clearly reduced the expression of VE-cadherin on the endothelial cell surface (31% and 24% respectively). Likewise, cell surface expression of β-catenin was reduced and severe gap formation between adjacent HUVECs developed. In cultured cells, β-catenin expression was mostly observed along the cell surface. Treatment with equimolar amounts of wtANGPT1 and ANGPT1 p.A119S failed to restore the reorganization of the F-actin cytoskeletal elements. ANGPT1 p.A119S variant in homozygous condition further diminished VE-cadherin and β-catenin expression and failed to reduce stress fibre formation significantly affecting the endothelial barrier functionality.

Conclusions and Clinical Relevance: Present data show that in a heterozygous state the p.A119S substitution results in a pathogenic loss of function of the protein due to a mechanism of haploinsufficiency. The ANGPT1 reduced ability to counteract the increment of endothelial permeability produced by inducers, such as VEGF and bradykinin, stimulate vascular leakage and reorganization of the F-actin cytoskeletal elements. As a result, a partial impairment of the ANGPT1 functionality, like when dominant mutations occur, represents a pathophysiological cause of HAE.

Angela Bruna Maffione and Maurizio Margaglione contributed equally to this work.

KEYWORDS
angiopoietin-1, endothelial barrier, gene mutation, haploinsufficiency, hereditary angioedema

1 | INTRODUCTION

Hereditary angioedema (HAE) is an autosomal dominant rare disorder characterized by episodic local subcutaneous and submucosal oedema. Physical signs of HAE include episodes of swelling of the skin and mucous membranes. The clinical expression is highly variable, from asymptomatic cases to patients suffering from disabling and life-threatening attacks with a demonstrated humanistic and economic burden.[1] The pathogenesis of HAE was extensively investigated and a series of loci were found to be causative of the disease. Most of the cases are due to mutations in the *SERPING1* gene (OMIM #106100). This gene codes for a serine protease, the C1 inhibitor (C1INH) and mutations in the *SERPING1* results in types I and II HAE.(1) In type I, representing 85% of patients, serum levels of C1NH are less than 35% of normal.[2,3] The type II is clinically indistinguishable from the type I and represents approximately 15% of cases. In type II, the levels are normal or elevated, but the protein is nonfunctional. The absence of adequate functional C1INH levels leads to an increased generation of bradykinin, a vasoactive peptide that causes increased vascular permeability by binding to the bradykinin B2 receptor. Another type of hereditary angioedema, HAE type III (OMIM #610618), is caused by mutation in the gene encoding for the coagulation factor XII (*F12*).[4] Recently, a mutation in the plasminogen gene was identified associated with HAE with normal C1INH functional levels and without mutations in the *SERPING1* or *F12* genes. Hereditary angioedema with a mutation in the PLG gene is a novel type of HAE (HAE-PLG).[5]

In a large Italian kindred with HAE and no mutation in the known causative genes, we have identified a missense variant (p.A119S) in the angiopoietin-1 gene (*ANGPT1*).[6] ANGPT1 promotes endothelial cell survival, inhibits vascular leakage and stabilizes normal mature vessels.[7-9] Bradykinin has been shown to be the key mediator of enhanced vascular permeability in HAE attacks. Pretreatment of endothelial monolayers with ANGPT1 attenuated the permeability-inducing effects of bradykinin.[10-12] In addition, ANGPT1 prevents VEGF-induced vascular permeability and facilitates vessel stabilization by maintaining endothelial cell adhesion.[13]

In family members carrying the ANGPT1 p.A119S variant, a significant impairment of the protein multimerization and capability to bind the natural receptor, tunica interna endothelial cell kinase 2 (TIE2), was shown. In the meanwhile, a further *ANGPT1* variant was identified in Brazilian HAE patients.[14,15] The identification of *ANGPT1* mutations causing HAE has an important implication for the pathogenesis of HAE, shifting the attention on a different way for the regulation of vascular permeability largely independent from bradykinin activity. *ANGPT1* variants appear to cosegregate with the clinical phenotype, indicating an autosomal dominant inheritance. Two different mechanisms can be supposed to explain the Mendelian inheritance observed: haploinsufficiency and a dominant negative effect.

Here, we investigated the mechanism by which the heterozygous *ANGPT1* p.A119S variant results in an increased vascular leakage. Based on our findings, we conclude that haploinsufficiency of ANGPT1 causes in HAE pathogenesis.

2 | METHODS

2.1 | Site-directed mutagenesis and transfection

Site-directed mutagenesis experiments were carried out to insert the identified mutation into angiopoietin-1(*ANGPT1*) (NM_001146) Human Tagged ORF Clone (Myc-DDK-tagged; OriGene, Rockville, Md) by using the QuikChange II Site-directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA), according to the manufacturer's instructions, as previously reported.[6]

Briefly, primer pairs containing the desired mutation and flanked unmodified sequence were used, and the presence of mutation in selected colonies was verified by means of sequencing.

HEK293 cells were transiently transfected with mutant or wild-type ANGPT1 plasmid using TurboFectin 8.0 Transfection Reagent, according to the manufacturer's instructions (OriGene, Rockville, Md, USA). The medium was harvested from transfected cells after 48 hours, and it was concentrated using a size-exclusion column (Amicon Ultra 10 kDa; Millipore, Temecula, CA, USA).

Recombinant proteins containing the FLAG sequence were purified by Clone OTI4C5, Anti-DDK (FLAG) Agarose beads (OriGene, Rockville, Md, USA). After purification, recombinant proteins were quantitated by using the Bradford assay.

2.2 | TIE2-Fc pulldown assay

WT and mutant ANGPT1 proteins were produced by transfection of HEK293 cells with expression vectors encoding FLAG-tagged variant proteins as described above.

Binding of the recombinant proteins to the soluble extracellular domain TIE2-Fc (Abcam, Cambridge, UK) was assayed by using an in vitro binding assay. Each recombinant protein (20 ng) and a mix of equal amount of WT and mutant recombinant protein that represent heterozygous condition was mixed with 100 ng of sTIE2-Fc and incubated in 500 mL of Tris buffer solution (50 mmol/L Tris and 100 mmol/L NaCl, pH 7.4) containing 0.02% Triton X-100 at 4°C for 2 hours. Then, 20 μL of Dynabeads Protein A (Thermo Fisher

Scientific, Waltham, Mass) was added and incubated for another 1 hour at 4°C. Dynabeads Protein A-conjugated samples were washed twice with 1 mL of Tris buffer containing 0.02% Triton X-100. The samples were eluted with Laemmli sample buffer containing β-mercaptoethanol and heat denatured and then separated further by using 10% SDS-PAGE, electroblotted onto nitrocellulose membranes. The presence of ANGPT1-TIE2-Fc complex was determined by Western blotting using Clone OTI4C5, Anti-DDK (FLAG) Monoclonal antibody, HRP.

2.3 | HUVEC stimulation assay

The ability of mutant ANGPT1 proteins, to stabilize the endothelial barrier function through VE-cadherin, was performed as follows: recombinant wt and mutant ANGPT1 were prepared as described above for the TIE-Fc pulldown assay. Passage four Human Umbilical Vein Endothelial cells (HUVECs) were seeded in 8-well chambers slide and cultured in EBM-2 endothelial growth basal medium (Lonza, Basel Switzerland) supplemented with 2% foetal bovine serum and SingleQuots™ Kit—growth factors, cytokines and supplements optimized for HUVECs(Lonza, Basel Switzerland). Twenty-four hours after seeding, HUVECs were serum starved for 16 hours and were then stimulated with recombinant normal ANGPT1 (wtANGPT1) and ANGPT1p.A119S variant (200 ng/mL, 30 minutes) VEGF (50 ng /mL, 15 minutes), bradykinin (1 μmol/L, 4 minutes). Co-treatments were performed directly into the wells (15 minutes with each recombinant protein followed by 15 minutes with VEGF or by 15 minutes with Bradykinin) and then fixed for immunocytochemistry analysis. Results from all studies were confirmed in at least three independent experiments.

2.4 | Immunocytochemistry

VE-cadherin and β-catenin distribution were determined by immunocytochemistry. Subconfluent HUVECs cells treated with VEGF and recombinant protein were seeded onto chamber slide. Treated cells were fixed (4% formaldehyde for 15 minutes at room temperature), permeabilized (0.1% Triton X-100 in PBS, pH 7.5, 10 minutes) and blocked (2% BSA in PBS, pH 7.5, 30 minutes) before staining. Cells were incubated with primary antibodies mouse anti-human VE-cadherin mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-human β-catenin (BD Biosciences San Jose, CA) overnight at 4°C and then with goat anti-mouse IgG-FITC antibody for 45 minutes at room temperature (Santa Cruz Biotechnology, Santa Cruz, CA, USA); nuclei were stained 4-6-diamidino-2-phenylindole-2HCl. Alexa 546-conjugated phalloidin (1:200) was used for staining for F-actin. Images were obtained using a Eclipse Ti-E, PFS and NIS Element, Confocal CD2 Time lapse (Nikon, Tokyo, Japan). Specimens were viewed through a 60X oil immersion objective, and images were acquired by the NIS-Elements Imaging Software (Nikon, Tokyo, Japan). The quantification of immunofluorescence intensity was performed with the NIS-Elements Imaging Software (Nikon, Tokyo, Japan).

Adherens junctions are represented by histograms of VE-cadherin (as indicated by lines) and were quantitatively analysed using peak fluorescence intensities of the histograms at the single-cell level for 10 cells/experiment (randomly selected).[15] ImageJ (NIH) was employed for data analysis.

2.5 | Immunoprecipitation and Western Blot

At the end of treatments, total cell lysates were harvested with HEPES lysis buffer with NP-40 containing 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 1% NP-40, 5 mmol/L EDTA, and 1× complete protease inhibitor mixture (Roche) and incubated with the VE-cadherin antibody and Dynabeads Protein G (Invitrogen). The beads were washed four times with PBS, boiled in SDS-PAGE loading buffer and resolved by SDS-PAGE. Membranes blotted with anti-phosphotyrosine antibody and VE-cadherin antibody to verify expression levels. Protein phosphorylation levels were normalized to the matching densitometric values of nonphosphorylated proteins.

3 | RESULTS

3.1 | Heterozygous ANGT1p.A119S conditioning of protein binding to TIE2 receptor

The ability of the mutant protein to bind the natural receptor was investigated by using a soluble form of the receptor (TIE2-Fc chimera protein). In order to distinguish between haploinsufficiency and a dominant negative effect, the heterozygous condition observed in plasma of HAE patients carrying an *ANGPT1* variant was mimicked using equimolar amounts of recombinant normal ANGPT1 (wtANGPT1) and ANGPT1p.A119S variant. In conditions reproducing the heterozygous state (Figure 1), in vitro binding assays of purified recombinant proteins revealed a reduction of the binding capability to the TIE2-Fc receptor (80,7%) that was further reduced in the homozygous condition (59,1%). These data were in agreement with those observed using native proteins from plasmas of HAE patients and point towards a mechanism of haploinsufficiency.[6]

3.2 | Heterozygous ANGT1p.A119S impairment on VE-cadherin and the β-catenin effect on vascular permeability upon VEGF treatment

The existence of an impairment of the barrier functionality of basal endothelial cells was assessed using HUVECs, a laboratory model system for study of endothelial cells.

In these experiments, we used VEGF, a well-known inducer of vascular permeability,[12] to test whether the ANGPT1 p.A119S variant may affect properties of endothelial cells to limit vascular permeability. The co-treatment of HUVECs with VEGF and recombinant wtANGPT1 prevented inter-endothelial gap formation and protected both the structural and functional barrier.

The molecule primarily responsible for cell-cell adhesions of ECs is the transmembrane homophilic adhesion molecule, vascular

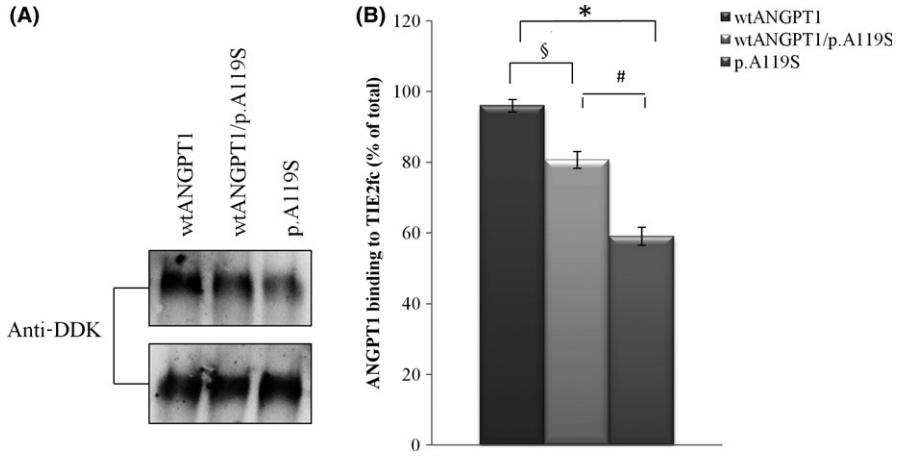

FIGURE 1 Heterozygous ANGT1p.A119S revealed a reduction of the binding capability to the TIE2-Fc receptor. A, Immunoblot for recombinant wtANGPT1, wtANGPT1/p.A119S and p.A119S variant bound to the soluble extracellular domain TIE2-Fc (upper panel) and total amounts assayed for pull down (lower panel). B, The ability of the wtANGPT1 and heterozygous A119S variant to bind TIE2-Fc was calculated after normalization for amounts assayed for binding and expressed as a percentage of the total value. Means and SEM of 3 experiments are shown

endothelial (VE)-cadherin.[16-18] Confocal microscopy analysis shows that the stabilization was reduced when endothelial cells were treated with recombinant ANGPT1 p.A119S or equimolar amounts of wtANGPT1 and ANGPT1 p.A119S. In vitro, conditions mimicking the heterozygous state showed a severe gap formation between adjacent HUVECs that was further evident when cells were treated with ANGPT1 p.A119S only (Figure 2A). Indeed, staining for VE-cadherin was intense and continuously distributed along the cell-cell borders of HUVECs treated with wtANGPT1. Whereas treatment with wtANGPT1 abolished the paracellular gaps formation (Figure 2A) induced by VEGF, the addition of ANGT1 p.A119S did not prevent it and the failure was increased when only the mutated ANGPT1 was used. Also, β-catenin plays an important role in endothelial cell-to-cell contact and prevents VEGF-induced permeability. The cytoplasmic domain of VE-cadherin binds to β-catenin, which is linked to the actin of the cytoskeleton.[18] Figure 2A shows the expression of β-catenin in the HUVEC cell model in order to assay the barrier functionality of endothelial cells. Confocal microscopy analysis revealed that, in the presence of VEGF, the addition of wtANGPT1 substantially restored β-catenin expression (Figure 2A). As shown in Figure 2, wtANGPT1 stimulated cells showed staining of β-catenin at HUVECs cell-to-cell points of contacts. Otherwise, the addiction to equimolar amounts of wtANGPT1 and ANGPT1 p.A119S reduces cell surface expression of β-catenin and shows severe gap formation between adjacent HUVECs. The only ANGPT1 p.A119S stimulation further diminished β-catenin expression. When co-cultured cells were stimulated with ANGPT1 p.A119S, β-catenin expression was mostly observed along the cell surface. Also, in laboratory conditions mimicking the heterozygous state, ANGT1p.A119S was not sufficient to restore endothelial cell-cell contact: staining for β-catenin was weak and punctate along the cell-cell borders of HUVECs. In VEGF-treated cells, the amount of VE-cadherin protein was significantly decreased (Figure 2C). wtANGPT1 significantly raised the amount of VE-cadherin protein. In contrast, equimolar amounts of wtANGPT1 and ANGPT1 p.A119S reduced fluorescence intensity. The amount of VE-cadherin protein was significantly further decreased when only the mutated ANGPT1 p.A119S was used.

3.3 | Heterozygous ANGT1p.A119 failed to prevent VE-cadherin and β-catenin redistribution induced by bradykinin

The ability of ANGPT1 to restrict the permeability increase in response to oedema-promoting agents has been previously described.[19] We investigated the effects of ANGPT1 p.A119S variant on enhanced vascular permeability induced by bradykinin (1 μmol/L). Consistent with its permeability effects, bradykinin drastically reduced the typical pattern of fluorescence localization of VE-cadherin (Figure 2B) at the cell-cell contacts. In bradykinin-treated cell, VE-cadherin immunostaining showed reduced amounts of VE-cadherin on cellular surface and several intercellular gaps between adjacent cells. Figure 2B shows that wtANGPT1 restore the integrity of the endothelial cell-cell junction. Co-treatment with equimolar amounts of wtANGPT1 and ANGPT1 p.A119S resulted in a marked change in the VE-cadherin staining. At cell borders, VE-cadherin were still detectable but with decreased expression. Preincubation with the only ANGPT1 p.A119S further lowered VE-cadherin expression. Bradykinin significantly disassembled VE-cadherin, whereas wtANGPT1 counteracted this effect (Figure 2C). Equimolar amounts of wtANGPT1 and ANGPT1 p.A119S reduced VE-cadherin fluorescence intensity, and the reduction was further increased when only the mutated ANGPT1 was used (Figure 2C).

We also evaluated the expression of β-catenin in HUVEC. Bradykinin drastically reduced the expression of β-catenin. The stimulation with wtANGPT1 restored the staining distribution. In cell culture pretreated with equimolar amounts of wtANGPT1 and ANGPT1

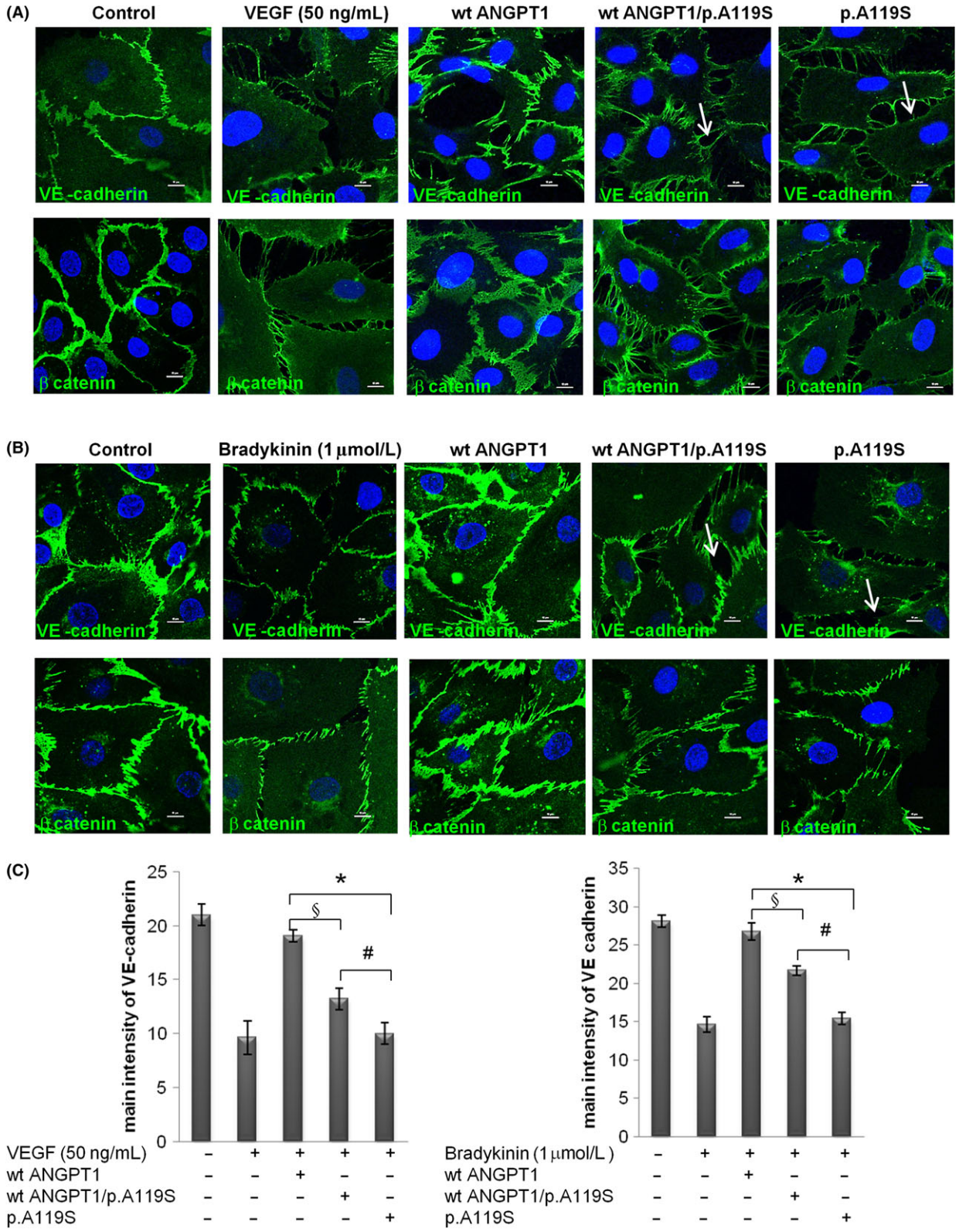

FIGURE 2 Heterozygous ANGT1p.A119S affects the VE-cadherin dependent vascular permeability upon VEGF (A) and bradykinin (B) treatment. Confocal fluorescent immunocytochemistry for VE-cadherin and β-catenin. Cells were incubated with VEGF (A) or bradykinin (B), and recombinant wtANGPT1, p.A119S and heterozygous wtANGPT1/p.A119S (200 ng/mL). The paracellular gaps formation were abolished in cells treated with wtANGPT1. White arrow highlights severe gap between adjacent HUVECs in heterozygous and homozygous condition. (Representative of 4 independent experiments) (scale bar = 10 μm). C, The effect of recombinant wtANGPT1, p.A119S and heterozygous wtANGPT1/p.A119S ANGPT1 was examined. Data from (A) and (B) were quantified using the NIS-Elements Imaging Software (Nikon, Tokyo, Japan). VE-cadherin (ie, intracellular intensity in arbitrary units) is shown

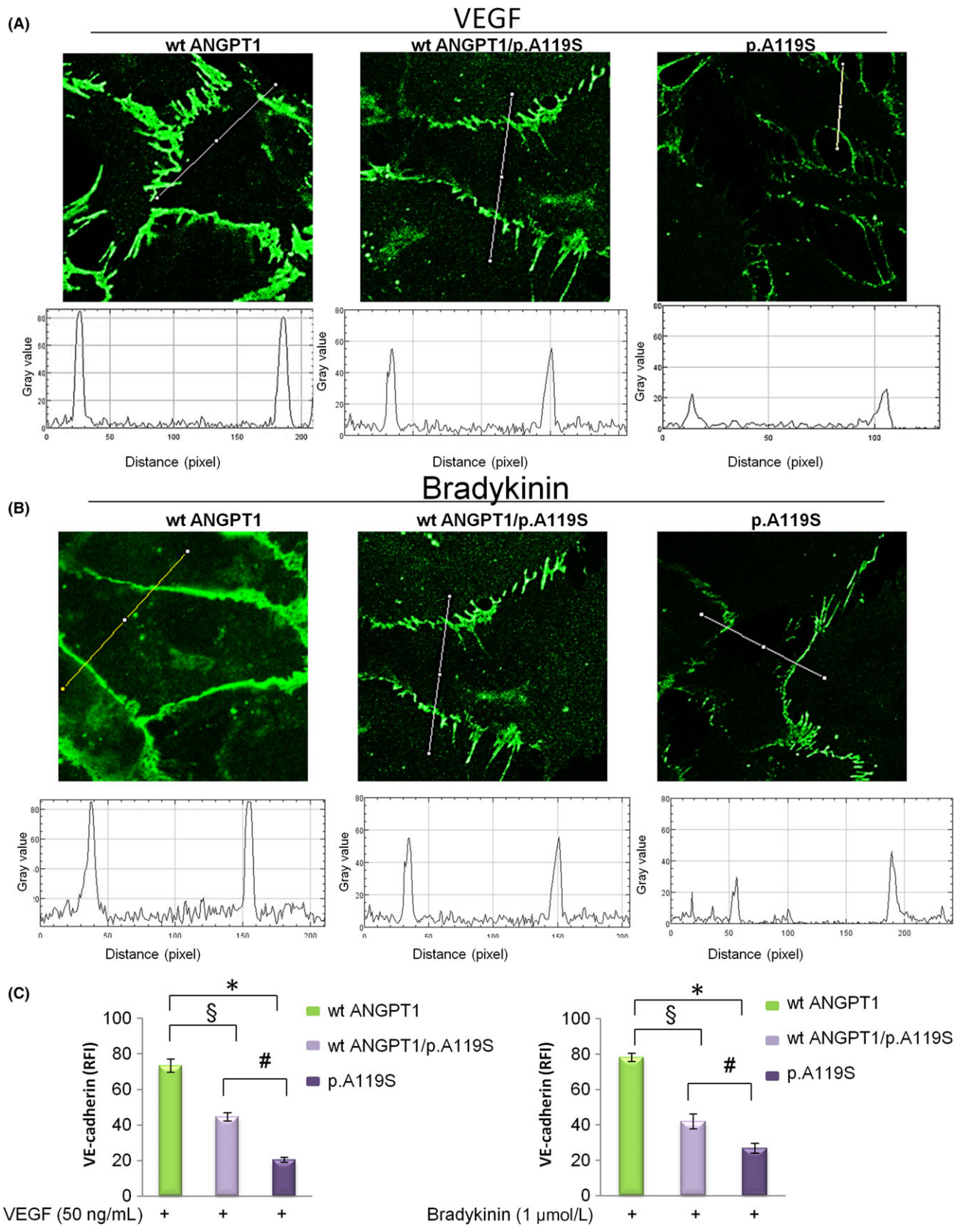

FIGURE 3 (A) Heterozygous wtANGPT1/p.A119S reduced VE-cadherin fluorescence intensity. (A) Representative images of VE-cadherin and adherens junctions (top) represented by VE-cadherin histograms (bottom), indicated by the dotted lines in the images. (B) Adherens junctions are represented by histograms of VE-cadherin (as indicated by lines) and were quantitatively analysed using peak fluorescence intensities of the histograms at the single-cell level for 10 cells/experiment (randomly selected). (C) 15 ImageJ (NIH) was employed for data analysis

p.A119S, confocal analysis of β-catenin distribution showed a discontinuous and punctate pattern of staining. ANGPT1 p.A119S failed to prevent β-catenin redistribution induced by bradykinin. (Figure 2B). The changes in the stability of VE-cadherin are represented by line profiles displaying the distribution of relative fluorescence intensity, as shown by dotted lines crossing two cell-cell contacts. VE-cadherin disassembly was also quantitatively analysed by measuring the peak fluorescence intensities of the histograms. (Figure 3).

3.4 | Heterozygous ANGT1p.A119 failed to prevent the VE-cadherin tyrosine phosphorylation

Tyrosine phosphorylation sensitizes VE-cadherin to the action of VEGF and bradykinin. As shown in Figure 4, the exposure of the endothelial cells to VEGF and bradykinin induced an VE-cadherin tyrosine phosphorylation detected within 15 minutes of stimulation. wtANGPT1 prevented this event. Quantification of normalized band intensity was shown in Figure 4. Heterozygous ANGT1p.A119 failed partially to prevent the VE-cadherin tyrosine phosphorylation induced by VEGF and bradykinin.

3.5 | Heterozygous ANGT1p.A119 failed to reduce VEGF and bradykinin-induced F-actin

Stress fibres are contractile actin bundles found in nonmuscle cells and play an important role in cellular contractility involved in cell adhesion.[19] Stimulation with VEGF (50 ng/mL) for 15 minutes or bradykinin (1 μmol/L) for 15 minutes generated a massive increase in stress fibres and redistribution of VE-cadherin (Figure 5). wtANGPT1 reduced significantly both VEGF and bradykinin-mediated alterations of endothelial filamentous actin (F-actin) and redistribution of vascular endothelial cadherin (VE-cadherin), as shown by double immunofluorescence. In contrast, pretreatment with equimolar amounts of wtANGPT1 and ANGPT1 p.A119S or isolated ANGPT1 p.A119S for 30 minutes before VEGF or bradykinin stimulation failed to reduce stress fibres formation. In homozygous condition, ANGPT1 p.A119S completely lacked to restore the organization of the F-actin cytoskeletal elements than the heterozygous condition (Figure 5).

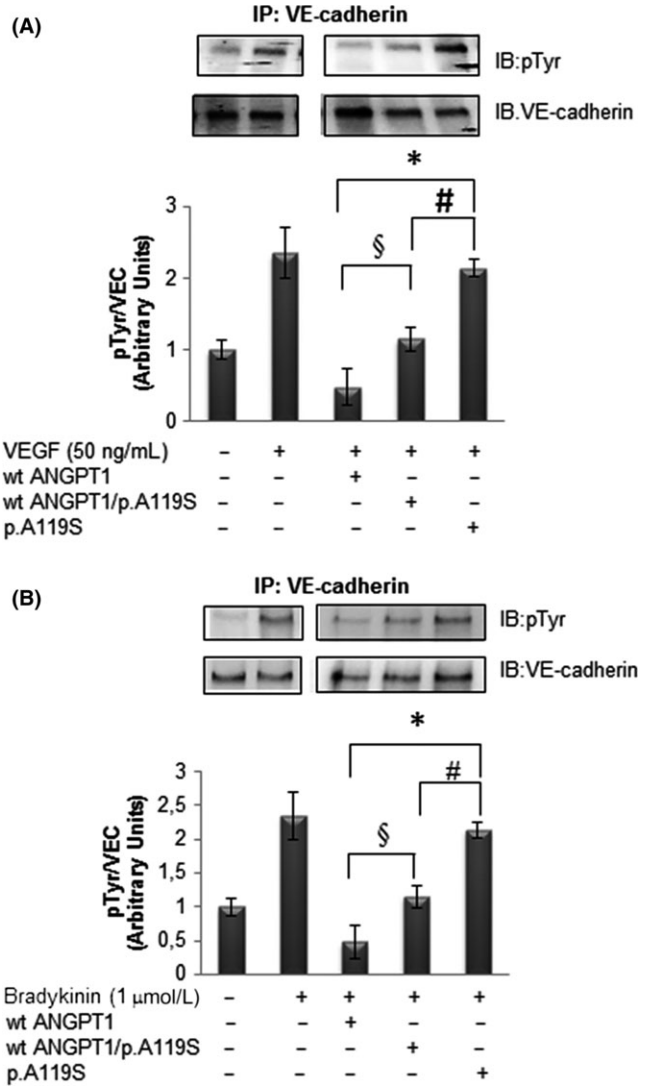

FIGURE 4 Heterozygous ANGT1p.A119 failed to prevent the VE-cadherin tyrosine phosphorylation. A, HUVECs treated with VEGF (50 ng/mL) and (B) bradykinin (1 μmol/L) were analysed for phosphotyrosinated-VE-cadherin in cell extracts. Cells were lysed and VE-cadherin immunoprecipitates analysed by immunoblots using anti-phosphorylated tyrosine or anti-VE-cadherin antibodies. Quantification of normalized bands intensity is shown

4 | DISCUSSION

Recent data land support to the hypothesis that pathways different from an impairment of the bradykinin regulation could be involved in HAE. The detection of missense *ANGPT1* variants suggests that vascular leakage may also occur when the effect of inducers, such as bradykinin and VEGF, is not counterbalanced. In HAE patients, the p.A119S variant was associated with reduced amounts of ANGPT1 in plasma and also impaired the ability of ANGPT1 to form multimers, leading to a reduction in the ability of the mutant protein to bind to the TIE2 receptor. Despite a series of findings support the effect of *ANGPT1* variants as a cause of HAE, no clear evidence shed light on the pathogenetic effect. Whereas animal models showed that a homozygous ANGPT1 deficiency produces early embryonic lethality,[20,21] a heterozygous state may imply, whether able to produce a clinical phenotype, either a mechanism of haploinsufficiency or a dominant negative effect. Present findings strongly suggest that haploinsufficiency is the mechanism by which the *ANGPT1* variant plays its role. Haploinsufficiency may occur through several ways. Heterozygous gene variants can lead to a partially nonfunctional protein with an impairment of protein functions although the total amount of that protein expressed in the cell is normal. The p.A119S variant affects the ability of the functional protein to assemble in the correct fashion. In in vitro conditions mimicking the heterozygous state, the p.A119S variant significantly reduced the capability to bind its natural receptor although clearly much less than the

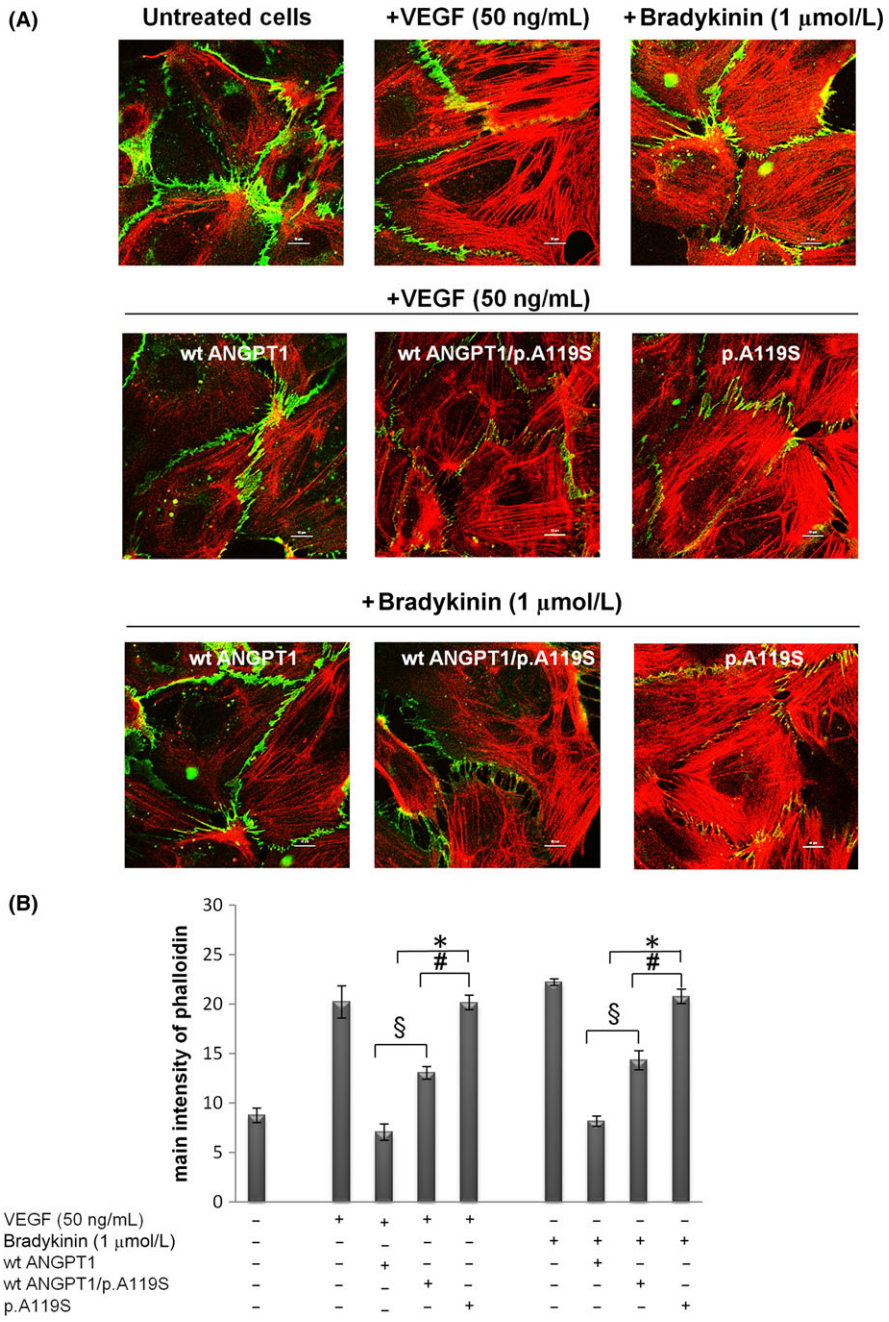

FIGURE 5 Heterozygous ANGT1p.A119S affects VEGF and bradykinin-induced F-actin and VE-cadherin redistribution. (A) HUVECs were grown to confluency on slides, stained for phalloidin and VE-cadherin, and visualized by double immunofluorescence (60X magnification). Cells control did not show stress fibres or intercellular gap formation as visualized with F-actin-specific phalloidin Alexa 546 (red) and VE-cadherin (green). VEGF and bradykinin 1 μmol/L caused a massive increase of stress fibres, redistribution of VE-cadherin and gap formation. Pretreatment with wtANGPT1 for 30 min before VEGF and bradykinin stimulation prevented VE-cadherin redistribution and gap formation, reduced stress fibres. Heterozygous wtANGPT1/p.A119S failed to prevent stress fibres formation. p.A119S shows a minor effect on restore reorganization of the F-actin cytoskeletal elements. Representative fields of HUVEC monolayers of 3 experiments are shown. (B) Data from (A) were quantified using the NIS-Elements Imaging Software (Nikon, Tokyo, Japan). VE-cadherin (ie, intracellular intensity in arbitrary units) is shown

homozygous condition. The next step was to assess whether the reduction of ANGPT1 was sufficient enough to affect the stabilization of endothelial barrier function. Findings from animal models and patients imply that the ANGPT1-TIE2 axis stabilizes the blood vascular endothelium and regulates barrier function.[9,22,24,25] In animal models, ANGPT1 regulates the integrity of the adult vasculature protecting from plasma leakage, particularly in inflammatory diseases.[23,24] Indeed, ANGPT1 contributes to the maintenance of endothelial barrier function, by inhibiting the effects of multiple permeability enhancing agents, including VEGF and bradykinin.[26,27]

It is widely accepted that the integrity of the endothelial cell barrier is critically upon cell junction and intact cytoskeletal structure. Bradykinin and VEGF induce cytoskeletal rearrangement and endothelial cell contractile response leading to the disruption of intercellular contacts and the increase of permeability.

The ability of ANGPT1 to restrict the increase of permeability in response to oedema-promoting agents has been previously described.[20,21] Bradykinin has been shown to be the predominant mediator of enhanced vascular permeability in HAE attacks. VEGF and ANGPT1 have similar effects on endothelial cell survival and proliferation but opposed effects on barrier function. At endothelial junctions, among variable cell adhesion molecules VE-cadherin plays a pivotal effect.[16] VEGF enhances endothelial permeability by promoting endocytosis of VE-cadherin.[13] This event is induced by phosphorylation of the VE-cadherin intracellular domain, which is protected by ANGPT1. Based on these findings, it is evident that an

imbalance due to a reduced ANGPT1 functionality in the presence of VEGF up-regulation can results in the enhancement of endothelial permeability and vascular leakage.

Present data show that in a heterozygous state the expression of VE-cadherin on the endothelial cell surface is strongly reduced. Confocal microscopic analysis revealed that this suppression occurs through endocytosis of VE-cadherin. The VE-cadherin is a transmembrane homophilic adhesion molecule and the cytoplasmic domain binds to-β-catenin,[17] which is linked to the actin of cytoskeleton. Linkage between VE-cadherin, β-catenin and the actin of cytoskeleton contributes to the formation of a strong adhesion. In heterozygous condition, staining for β-catenin was weak and punctate along the cell-cell borders of endothelial cells suggesting that the ANGT1 p.A119S variant is not sufficient to restore endothelial cell-cell contact. In addition, present data show that heterozygous ANGPT1 p.A119S failed to reduce stress fibre formation induced by both VEGF and bradykinin, although in homozygous condition ANGPT1 p.A119S failed to restore the organization of the F-actin cytoskeletal elements than the heterozygous condition.

In conclusion, the p.A119S substitution results in a pathogenic loss of function of the protein due to a mechanism of haploinsufficiency. As a result, the ANGPT1 reduced ability to counteract the increment of endothelial permeability produced by inducers, such as VEGF and bradykinin, causes vascular leakage. We recognize that a partial impairment of the ANGPT1 functionality, like when dominant mutations occur, represents a pathophysiological cause of HAE.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

RS, ALC and GC performed the experiments, analysed data, and reviewed the manuscript. ABM and MM organized the study and supervised the experiments. MD designed the project and wrote the manuscript.

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