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Preserving vascular stability: FVIII's influence on endothelial cells

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SSD: BIO/17

« [El envestigator es] ministro del progreso, sacerdote de la verdad y confidente del Creador. Él acierta exclusivamente a comprender algo de ese lenguaje misterioso que Dios ha escrito en la naturaleza, y a él solamente le ha sido dado desentrañar la maravillosa obra de la Creación para rendir a lo Absoluto el culto más grato y acepto, el de estudiar sus portentosas obras, para en ellas y por ellas conocerle, admirarle y reverenciarle.»

(Ramon y Cajal, 1852-1934)

Student declaration

I declare that the PhD thesis titled "Preserving vascular stability: FVIII's influence on endothelial cells" represents my own work and that it has not been submitted, in whole or in part, in any previous application for a degree. I generated and analyzed the data herein reported. The following experiments have been conducted in collaboration:

- RNAseq data analysis was performed in collaboration with Prof. Ivan Molineris and Dr. Francesca Anselmi (Epigenetics Lab, Dept. of Life Science and System Biology, Università di Torino)
- Animal procedures were performed by Dr. Cristina Olgasi and Dr. Chiara Borsotti (Histology Lab, Dept. of Translational Medicine and Health Sciences respectively, Università del Piemonte Orientale)
- Guides for CRISPR/Cas9 were designed and selected by Paola Capasso and Angelo Lombardo (San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milano)
- Proteomic data analysis was performed in collaboration with Prof. Marcello Manfredi (Proteomic Lab, Dept. of Translational Medicine, Università del Piemonte Orientale)
- Oroboros data were generated in collaboration with Dr. Simone Reano (Biochemistry Lab, Settore Centri di Ricerca e Infrastrutture Polo NO, Università del Piemonte Orientale)

This PhD thesis is intended as confidential since the work or part of it, is not submitted for publication yet. Some of the results reported in this PhD thesis are currently under revision.
The work done in my PhD program is not exclusive of this thesis, but I contributed to the following publications:

- "Factor VIII promotes angiogenesis and vessel stability regulating extracellular matrix proteins". Haematologica. 2024. C. Olgasi*, A. Cucci*, I. Molineris*, **S. Assanelli***, F. Anselmi, C. Borsotti, C. Sgromo, A. Lauria, S. Merlin, G. E. Walker, S. Oliviero, A. Follenzi
- "GP64-pseudotyped lentiviral vectors target liver endothelial cells and correct hemophilia A mice". EMBO Molecular Medicine. M. Milani, C. Canepari, **S. Assanelli**, S. Merlin, E. Borroni, F. Starinieri, M. Biffi, F. Russo, A. Fabiano, D. Zambroni, A. Annoni, L. Naldini, A. Follenzi, A. Cantore

- "Hemostasis and endothelial functionality: the double face of coagulation factors". Haematologica. 2024. C. Olgasi*, **S. Assanelli***, A. Cucci*, A. Follenzi
- "Formation of Re-Aggregated Neonatal Porcine Islet Clusters Improves In Vitro Function and Transplantation Outcome". Transplant International. 2022. M Honarpisheh, Y Lei, Y Zhang, M Pehl, E Kemter, M Kraetzl, A Lange, E Wolf, L Wolf-van Buerck, J Seissler, **VANGUARD consortium**
- "Organoids: a systematic review of ethical issues". Stem Cell Research & Therapy. 2022. Dide de Jongh, Emma K Massey, **VANGUARD consortium**, Eline M Bunnik

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DECLARATION AND AUTHORISATION TO ANTIPLAGIARISM DETECTION

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Date: 23/04/2024

Signature: Simone Assanelli

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Abstract (english)

Hemophilia A (HA) is a genetic bleeding disorder caused by the deficiency of coagulation factor VIII (FVIII) and its severity is categorized based on FVIII residual activity: severe (< 1%), moderate (1-5%), and mild (5-40%). Current treatment primarily consists of frequent FVIII infusions, aiming to prevent spontaneous hemorrhages. These bleeding episodes are HA patients' hallmarks, but they also manifest hemarthrosis and spontaneous intracranial hemorrhages (ICHs) whose exact cause remains unclear. Furthermore, HA patients exhibit endothelial dysfunction and a pro-inflammatory environment, suggesting the potential involvement of FVIII in maintaining endothelial cell (EC) homeostasis.

To explore the role of FVIII in endothelial stability, we investigated the functionality of blood outgrowth endothelial cells obtained from both healthy (C-BOECs) and severe HA individuals (HA-BOECs). HA-BOECs showed a reduced tubulogenic capacity, decreased migratory ability, and a higher permeability than C-BOECs. Interestingly, when HA-BOECs were transduced with a lentiviral vector (LV) carrying FVIII or when treated with recombinant FVIII, they displayed rescued functions. To corroborate these data, we also performed CRISPR/Cas9 knockout of *F8* in C-BOECs showing a significantly decreased *in vitro* functionality.

For the first time, we found that FVIII binds to integrin βeta1 (ITGB1) activating focal adhesion kinase (FAK) and its downstream effectors in ECs. Moreover, both transcriptomic and proteomic analysis demonstrate that BOECs expressing FVIII result in a higher expression of genes and proteins related to angiogenesis, and extracellular matrix organization. Specifically, we focused on nidogen 2, involved in the endothelial basement membrane stability, whose expression was dependent to FVIII-ITGB1-FAK pathway.

Subsequently, we found a drastic reduction in vascular density in HA brains compared to WT ones, and, importantly, long-term LV-FVIII injected HA mice significantly improved brain vessel formation. These preliminary data indicate that FVIII plays a critical role in the brain vasculature development.

In conclusion, we explored the intricate effects of FVIII on ECs demonstrating its direct role in preserving the physiological functions of these cells. In HA, where FVIII levels are reduced or absent, disruptions in endothelial signaling occur, potentially leading to vessel fragility. This study is central to formulating precise therapeutic strategies for HA patients.

Abstract (italiano)

L'emofilia A è una malattia emorragica genetica causata dalla mancanza del fattore VIII (FVIII). Il trattamento prevede frequenti infusioni di FVIII, che hanno lo scopo di prevenire episodi emorragici spontanei. Questi sanguinamenti sono tipici dei pazienti emofilici, i quali, però, soffrono anche di emartrosi e spontanee emorragie intracraniche di cui non si conosce la causa. Inoltre, alcuni dati clinici riportano una significativa disfunzione endoteliale e un profilo pro-infiammatorio in questi pazienti, suggerendo un potenziale ruolo del FVIII nell'omeostasi delle cellule endoteliali.

Nella nostra ricerca, innanzitutto, abbiamo osservato che la funzionalità delle cellule endoteliali isolate dal sangue di pazienti emofilici gravi (HA-BOECs) è notevolmente ridotta rispetto a quella delle cellule di soggetti sani (C-BOECs) in termini di formazione di tubuli, migrazione e permeabilità. Inoltre, tali funzioni possono essere ripristinate aggiungendo il FVIII nelle cellule emofiliche o in acuto o attraverso terapia genica. Questi dati sono stati confermati riducendo l'espressione del FVIII nelle cellule sane grazie a CRISPR/Cas9 e osservando che una ridotta funzionalità. Per la prima volta, mostriamo che la capacità del FVIII di regolare queste funzioni è data dal legame con l'integrina beta 1 (ITGB1), legame che attiva la fosforilazione di *focal adhesion kinase* (FAK) e una serie di effettori intracellulari. Inoltre, sia le analisi trascrittomiche che proteomiche dimostrano che le cellule endoteliali che esprimono FVIII mostrano anche una maggiore espressione di geni e proteine legati all'angiogenesi e all'organizzazione della matrice extracellulare. In particolare, ci siamo concentrati sul nidogeno 2, la cui espressione è dipendente dal pathway FVIII-ITGB1-FAK.

Successivamente, abbiamo riscontrato una riduzione della densità vascolare nei cervelli di topi emofilici rispetto ai sani, e abbiamo osservato che topi emofilici trattati con la terapia genica mostravano un aumento significativo nella formazione dei vasi cerebrali. Questi dati suggeriscono che il FVIII svolge un ruolo cruciale nello sviluppo della vascolarizzazione cerebrale.

In conclusione, in questo lavoro di tesi abbiamo esplorato gli effetti del FVIII sulle cellule endoteliali dimostrando il suo diretto ruolo nel mantenere un fenotipo fisiologico di queste cellule. Questa ricerca è utile per la formulazione di più precise strategie terapeutiche per i pazienti con l'emofilia A.

Introduction

1. Coagulation factor VIII and hemophilia A

a. Structure and function of coagulation factor VIII

The gene that encodes factor VIII (*F8*) resides on the long arm of the X chromosome (Xq28). It measures 186 kb and it comprises 26 exons and 25 introns. The largest exon is exon 14 (3106 bp) encoding for the B domain of factor VIII (FVIII). Consequently, the resultant mRNA measures approximately 9 kb, with a coding sequence spanning 7053 bp. *F8* encodes for a mature inactive protein of 2332 amino acids (263 KDa) and is structured into six domains: A1, A2, B, A3, C1, and C2 (Figure 1A) (Vehar et al. 1984, Pipe 2009, Pablo-Moreno et al. 2022).

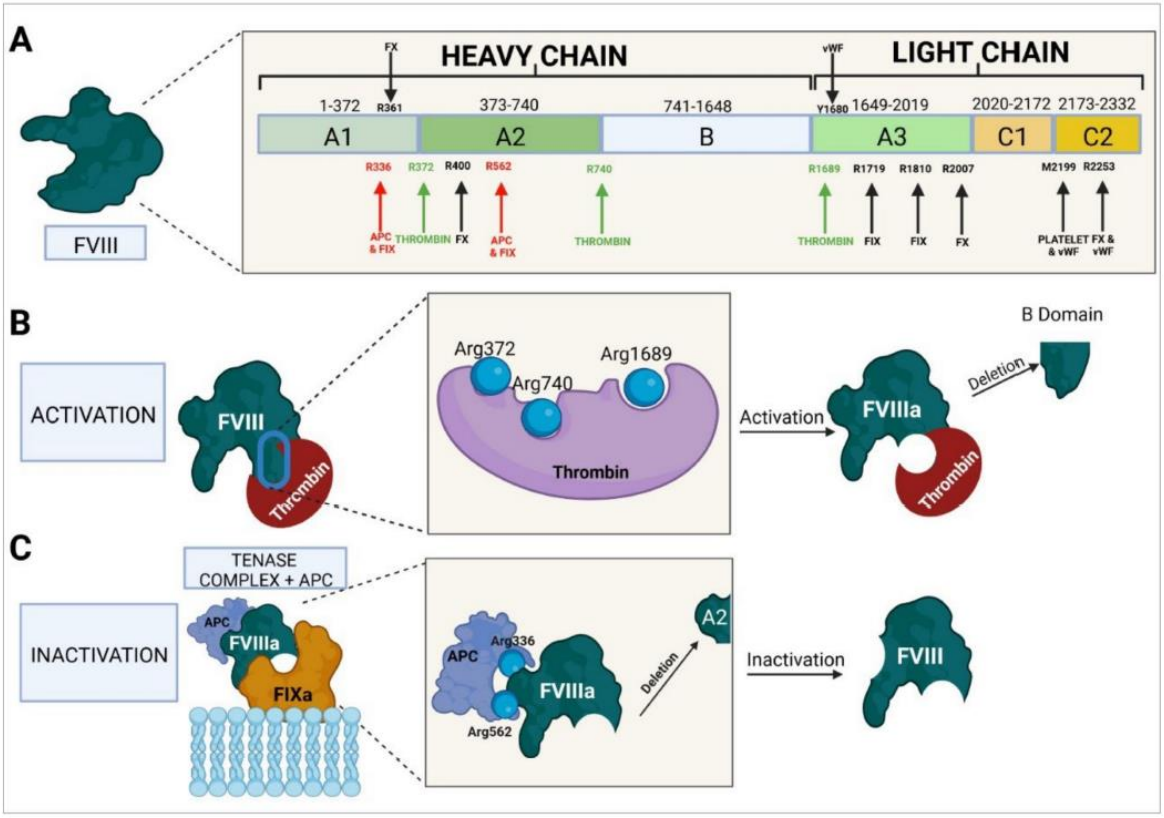


Figure 1. Molecular features of factor VIII. (A) FVIII domains, residues with which it interacts with other factors, and activation and inactivation sites. (B) Activation and B domain deletion thought thrombin action. (C) Inactivation and A2 domain deletion through APC. FVIII, factor VIII; APC, activated protein C; FIXa, activated factor IX; FX, factor X; vWF, von Willebrand factor. (Pablo-Moreno et al. 2022)

Following cleavage by the furin protease, FVIII is divided in a heavy chain weighing 200 kDa (A1-A2-B) and a light chain of 80 kDa (A3-C1-C2) (Figure 1A). Subsequently, FVIII needs to be activated by thrombin which binds to FVIII residues Arg372, Arg740 and Arg1689 cleaving B domain (Figure 1B).

Through *in silico* computational analyses and *in vitro* molecular experiments, the functions of the majority of FVIII domains have been elucidated, with the exception of the B domain (D'Oiron et al. 2004, Venkateswarlu 2014, Shearin and Venkateswarlu 2017, Dagil et al. 2019). The B domain, encoded by a single extensive exon, is positioned approximately at the midpoint of the entire protein (amino acids 741-1648) (Pipe 2009). Its sequence lacks homology with other proteins, including the B domain of factor V (FV), despite some similarities shared between these two factors (Pablo-Moreno et al. 2022). Additionally, B domain crystal structure is not available in the Protein Data Bank (PDB). Although it has been demonstrated that it plays a pivotal role in the regulation of intracellular trafficking, secretion and clearance of FVIII (Pipe 2009), most of the data suggest that B domain is not essential for the coagulative activity of this protein. Indeed, activated FVIII (aFVIII) is composed of a trimeric protein structure comprising an A1 domain (amino acids 1–372) and an A2 domain (amino acids 373–740), collectively constituting the heavy chain with a weight of 92 kDa. Additionally, it includes the A3, C1, and C2 domains (amino acids 1690–2332), which form the light chain weighing 80 kDa.

In vivo, aFVIII binds to the phospholipid surface (PS) through C1 and C2 domains (Novakovic et al. 2011, Orfeo et al. 2016) and initiates its effect on activated factor IX (FIXa) within a complex that triggers the activation of factor X (FX) (Eaton et al. 1986, Fay 2004). aFVIII binds FIXa through amino acids 336, 558–565, 1810–1818, and 1719, forming the tenase complex and to FX through amino acids 361–363, 400–409, 2007–2016, and 2253–2270 (Jenkins et al. 2004, Takeyama et al. 2018). These events lead to the continuation and the propagation of the coagulation cascade which, finally, brings to the conversion of fibrinogen to fibrin and to the subsequent clot formation. aFVIII is subsequently inactivated by activated protein C (APC) or by aFX which bind to Arg336 and Arg562 amino acids cleaving A2 domain (Shen and Dahlbacks 1994, Plantier et al. 2010) (Figure 1C).

In the bloodstream, FVIII is complexed with von Willebrand factor (vWF), a chaperone molecule which increases the half-life of FVIII by reducing its clearance and its binding with APC. When vascular injury occurs, vWF binds to exposed collagen at the site of injury,

promoting platelet adhesion and aggregation. This positioning of vWF-bound FVIII initiates the coagulation process by facilitating interaction with activated platelets, thereby enhancing the catalytic activity of FIX and subsequently activating FX (Terraube et al. 2010, Pablo-Moreno et al. 2022).

b. Hemophilia A: etiology and epidemiology

Hemophilia is a bleeding disorder that disrupts the typical hemostatic processes in the body. There are two main types of hemophilia: hemophilia A (HA), caused by a deficiency or inadequate levels of functional FVIII in the bloodstream, and hemophilia B (HB), which arises when there is a mutation in functional FIX (Castaman and Matino 2019). HA stands as the most prevalent disorder, affecting approximately 1 in 5000 male live births (Bolton-Maggs and Pasi 2003, Iorio et al. 2019). Indeed, the inheritance pattern of HA is linked to the X chromosome. Consequently, males carrying a defective copy of the *F8* on their X chromosome will pass it only to their daughters who will become carriers while their sons will be unaffected. Surprisingly, approximately one-third of hemophilia cases manifest sporadically, arising from spontaneous mutations, without any prior indication within the family history (Goodeve and Peake 2003, Oldenburg et al. 2004).

According to the international database updated in March 2022, 3052 unique variants in *F8* exist (https://f8-db.eahad.org/) and most of the HA patients live in the United States and in Europe, especially in Italy (Figure 2).

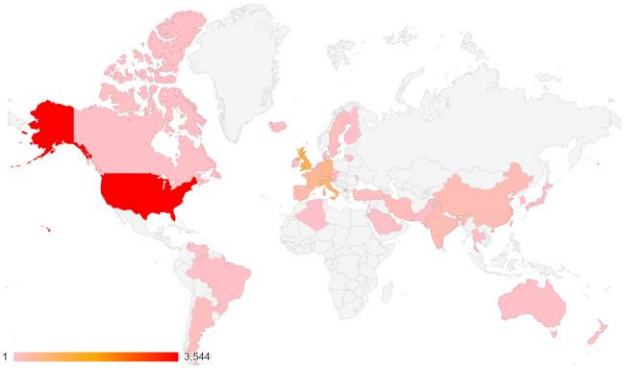


Figure 2. Distribution of HA patients reported from different parts of the world. The data represented in the figure are based on the cases reported in the international database alone (https://f8-db.eahad.org/world_map.html.php). HA, hemophilia A.

With a slight difference, the CDC Hemophilia A Mutation Project (CHAMP) identified 3756 unique variants (update to June 2020) (https://abstracts.isth.org/abstract/the-cdc-hemophilia-a-mutation-project-champ-f8-mutation-list-2020-update/). What is certain is that the most common mutation, observed in nearly 50% of individuals with the severe form of HA, involves the inversion of the intron 22 exons of *F8* (Int22Inv), resulting in an inversion of exons 1 to 22 compared to exons 23 to 26 and no functional circulating FVIII (Lakich et al. 1993, Naylor et al. 1993). Other mutations in *F8* are missense changes (46.3%), followed by frameshift (23.8%), nonsense (11.2%) and mutations with lower frequency as splice site change (8.5%), large structural change (5.8%), small structural change (2.3%) and others (2.1%) (Figure 3).

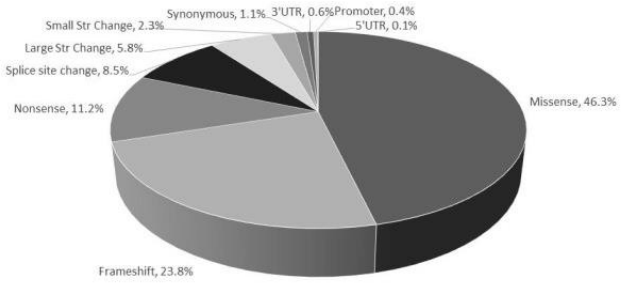


Figure 3. Frequency of *F8* variant types in the CHAMP mutation list. These frequency listed in CHAMP database represents worldwide HA patients updated to June 2020. (https://www.cdc.gov/ncbddd/hemophilia/champs.html) CHAMP, CDC Hemophilia A Mutation Project; HA, hemophilia A.

2. Current treatment for hemophilic A patients

To date, there is no definitive cure for HA, and the standard therapeutic approach involves replacement therapy with recombinant factor VIII (rFVIII), administered via intravenous injections as required to manage acute hemorrhages or according to prophylactic regimens to prevent bleeding episodes (Collins et al. 2009, Franchini and Liumbruno 2019). The regular long-term prophylaxis prevents bleedings as well as joint deterioration and chronic arthropathy which could lead to permanent cartilage injury. This treatment also reduces mortality preventing fatal hemorrhages. For patients with moderate or mild bleeding phenotypes, treatment may be given as needed, with the factor being administered when a bleeding episode occurs. However, for those with severe bleeding phenotypes, treatment must be provided regularly and

continuously (Srivastava et al. 2020, Miesbach and Eladly 2021, Lewandowska et al. 2022, Ozelo and Yamaguti-Hayakawa 2022a). Although the prophylaxis has reached great outcomes, the adherence of the patients is still challenging mainly due to the frequency of the intravenous infusions (Thornburg and Duncan 2017). Indeed, the half-life of the FVIII is around 10-12 hours but many improvements of the native molecule of FVIII have been performed during the last decades.

New generations of rFVIII has been developed through enhancements of the recombinant protein, which involve refining crucial post-translational modifications such as glycosylation. These modifications aim to enhance the stability of the mature FVIII protein (Thornburg and Duncan 2017). Additionally, advancements include the implementation of a covalent link between the FVIII heavy and light chains which safeguards the FVIII molecule from premature degradation and enhances its binding affinity for VWF (Zollner et al. 2014, Mahlangu et al. 2016). These molecules result in extended half-life (EHL) products and, thus, in an amelioration of the lifestyle of the patients (Zollner et al. 2014, Mahlangu et al. 2016). Another way to enhance the half-life of FVIII is the conjugation with polyethylene glycol (PEGylation) (Santagostino et al. 2020) or fusion with Fc portion of immunoglobulin (Mahlangu et al. 2014), both resulted safe and effective. Many PEGylated rFVIII products are now used in clinic, such as rurioctocog alfa pegol but are not generally used in children under 12 because of the lack of certainty regarding their long-term safety in this age range.

EHL product	Generic name	Mechanism of action	Half-life of EHL product (hours)	Half-life of standard factor (hours)	Frequency of administration
FVIII extended half-life products for use in Haemophilia A					
Lonoctocog alfa pegol	Afstyla	B-domain truncated single chain	14.5	13.3	Every second day
Efmoroctocog alfa	Elocta	FVIII Fc fusion protein	19.0	12.4	Effective with once-weekly administration
Damoctocog alfa pegol	Jivi	PEGylated B-domain deleted FVIII	18.5	13.0	Once weekly administration possible
Rurioctocog alfa pegol	Adynovi	PEGylated FVIII	14.3	10.4	Twice weekly or less
Turoctocog alfa pegol	NovoEight	PEGylated B-domain truncated FVIII	19.5	13.0	Every 4–7 days

Table 1. Comparison of different rFVIII EHL products with standard replacement therapy. This table summarize available EHL products for HA, their mechanism of action, half-life and frequency of administration. rFVIII, recombinant factor VIII; EHL, extended half-life; HA, hemophilia A. Adapted from (Mahlangu et al. 2014)

EHL products have demonstrated significant potential as substitutes for standard factor replacement decreasing the frequency of administration. Nonetheless, there is no direct comparison among various EHL products because they have been evaluated only against their respective standard factor counterpart and this makes it challenging to draw conclusive comparisons regarding their efficacy (Okaygoun et al. 2021). Future research should focus on comparing different EHL products to optimize the treatment of HA.

Despite these great improvements, replacement therapy still shows an important limitation and it cannot be considered a definitive cure. Indeed, 5-30% of HA patients receiving FVIII develop neutralizing inhibitor antibodies to FVIII making the treatment ineffective (Varthaman and Lacroix-Desmazes 2019). For this reason, a novel class of molecules has been using to treat HA patients, called non-replacement therapies because FVIII is not contained in these products. One of them is a monoclonal antibody called Emicizumab, a bispecific antibody which mimics the co-factorial activity of FVIII bridging activated FIX and FX (Kitazawa et al. 2012) (Figure 4). Other advantages of this molecule are subcutaneous delivery and longer duration of action. Indeed, Emicizumab can be administered once every 2-4 weeks reducing bleeding episodes and, improving patients' care and adherence (Pipe et al. 2019).

Other compounds which are part of the non-replacement therapy are the so-called rebalancing agents which consist in systemic antifibrinolytics or inhibitors of the coagulation cascade such as anti-APC and anti-tissue factor pathway inhibitor (anti-TFPI). To date, no rebalancing agents are approved and their efficacy is being tested in clinical trials (Mancuso et al. 2021, Ozelo and Yamaguti-Hayakawa 2022b, Pishko and Doshi 2022). The debate on which therapeutic approach for HA patients is the best is still open as suggested by the provocative title of an editorial published on Blood Transf. on 2019 claiming: "*'The times they are a-changin', ⋯ Or maybe not?*" (Franchini and Liumbruno 2019).

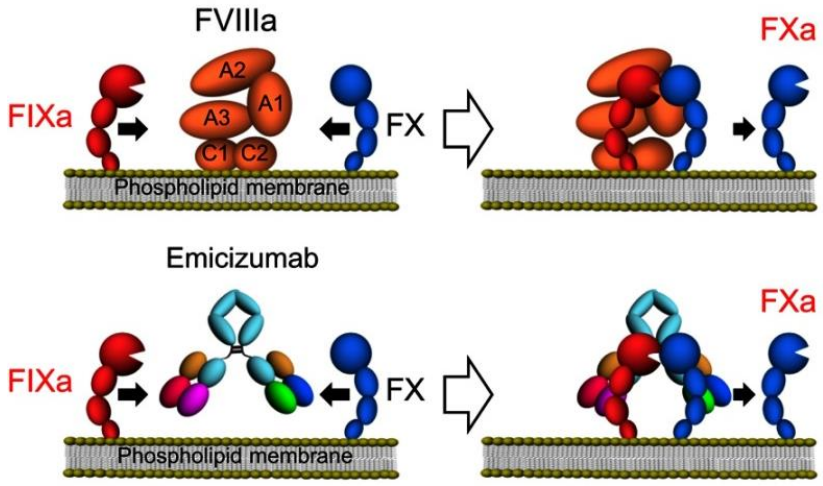


Figure 4. Mechanism of action of FVIII and Emicizumab. aFVIII positions aFIX and FX advantageously in space, facilitating and increasing the activation of FX by aFIX. Emicizumab acts similarly, mimicking the action of aFVIII. FVIII, factor VIII; aFVIII, activated FVIII; aFIX, activated factor IX; FX, factor X. (Yada and Nogami 2019)

3. Gene therapy for hemophilia A

Gene therapy represents a promising therapeutic strategy for monogenic diseases such as HA which emerges as an optimal candidate for this approach also because the restoration of FVIII levels from severe to moderate is adequate to improve the bleeding symptoms experienced by patients, leading to an overall enhancement in their quality of life. Thus, gene therapy continues to be a focal point of research due to its potential for significant outcomes in HA and other monogenic disorders. Its primary aim is to safely and effectively introduce genetic material into target cells to achieve therapeutic effects or slow down disease progression. The challenge is the development of one or more systems capable of efficiently transferring genes into selected tissues without inducing pathological consequences in patients. The genetic material must be delivered via a vector and target the appropriate cell types for the pathology. The goal of this approach is to enable cells to autonomously express the candidate gene for a prolonged period of time (Verma and Weitzman 2005, Dunbar et al. 2018).

Two types of vectors exist: viral and non-viral. Non-viral vectors seem to be less efficient than viral ones and, for this reason, less used. Recently, a new study showed a safe and efficient correction of HA in a mouse model through a non-viral ultrasound-mediated delivery of *F8* (Song et al. 2022). Other non-viral approaches consist in the use of liposomes and nanoparticles

but the data are scarce and more studies are needed (Liras and Olmedillas 2009, Zangi et al. 2023). To date, viral vectors remain the predominant choice in clinical applications due to their efficiency and safety in gene transfer. Their great advantage consists in the exploitation of the innate capability of viruses to deliver genes into the host cell, while excluding their ability to replicate and cause disease. Additional safety measures have been implemented to prevent homologous recombination between shared sequences that could potentially reconstruct the original viral genome. Various viral vectors have been engineered with DNA or RNA genomes and with or without integrating capabilities. Adeno-associated vectors (AAVs) are predominantly utilized as non-integrating vectors in clinical settings, whereas retroviral and lentiviral vectors (LV) are commonly employed among integrating vectors (Verma and Weitzman 2005, Zangi et al. 2023).

Unlike viral vectors, which are limited to promote only gene addition, new genome editing technologies have the capability to perform various modifications beyond gene addition such as gene ablation, gene correction and other highly targeted genome alterations within cells (Figure 5). Genome editing can be conducted on cells *ex vivo*, or the editing machinery can be administered *in vivo* to accomplish *in situ* genome editing (Dunbar et al. 2018). Thus, the goal of gene therapy for HA is to generate products as single-dose treatments capable of prolonged FVIII expression at therapeutic levels. This would eliminate the necessity for patients to undergo regular intravenous infusions.

In addition and in combination with gene therapy, cell therapy has been described as a new approach to increase and maintain the expression of a specific gene of interest. It consists on the infusion or transplantation of cells from external sources into a patient to treat a particular disease. In the context of genetic diseases, cell therapy approaches aim to transfer the correct gene into cells that will be transplanted, offering an alternative strategy to conventional gene therapy.

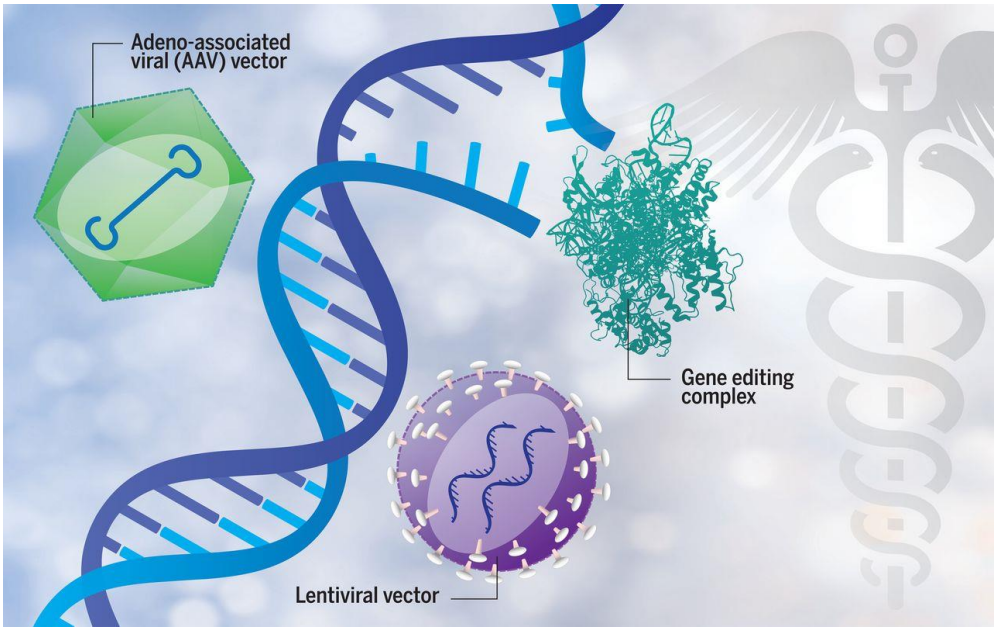


Figure 5. Gene therapy through adeno-associated vectors (AAV), lentiviral vectors (LV) and gene editing tools. AAV and LV are well-known gene therapy approaches which have been widely studied and used in clinical trials. On the other hand, gene editing complexes are recent technologies but anticipated to assume a progressively significant role in the field of gene therapy. (Dunbar et al. 2018)

a. Why endothelial cells are a good target for gene and cell therapy in hemophilia A

Designing a gene- or cell-based therapy requires identifying the optimal cell sources for treating the disease. The debate surrounding the synthesis site of FVIII has persisted for decades. Numerous studies indicate that the liver is the primary site for producing circulating FVIII. About 50 years ago, it was already demonstrated that the transplantation of healthy livers both in dogs and in human subjects restored the circulating FVIII levels comparable to physiological values (Marchioro et al. 1969, Bontempo et al. 1987). The discovery of four specific hepatocyte transcription factors (TFs) binding to the *F8* promoter also corroborated the hepatic origin of FVIII (Figueiredo and Brownlee 1995). However, it has been discussed for several years about the FVIII specific expression (Wion et al. 1985, Zelechowska et al. 1985, Do et al. 1999, Becker et al. 2004). Nowadays, it is well established that liver sinusoidal endothelial cells (LSECs) are the main source of FVIII in the human body (Shahani et al. 2014, Zanolini et al. 2015a). Moreover, *in silico* analysis of the *F8* promoter demonstrated that it contains also typical endothelial and hematopoietic TFs binding sites corroborating its endothelial expression (Merlin

et al. 2019a). Two TFs have been widely studied in the transcription regulation *F8*: Ets-1 and Ets-2. Using a luciferase reporter gene under the control of the *F8* promoter, they examined the *F8* promoter's activity. These findings revealed an increase in *F8* promoter (pF8) activity in the presence of Ets-1 alone, and even stronger activity in the presence of both Ets-1 and Ets-2. However, Ets-2 alone did not affect pF8 activity (Famà et al. 2021). Ets-1 and 2 are members of the Ets TF family, known for their pivotal role in the survival of embryonic endothelial cells and adult angiogenesis. These results helped in a greater comprehension of pF8 regulation and identified the minimal and functional pF8 portion safeguarding its specificity and reducing the size of the plasmid, thus, allowing new gene therapy approaches.

Beyond LSECs, plasma FVIII may originate from additional sources, e.g., lymphatic endothelial cells, peripheral blood mononuclear cells, blood outgrowth endothelial cells (BOECs), microvascular endothelial cells derived from lung, kidney, heart, spleen, intestine, and skin (Shahani et al. 2010).

Considering these data, endothelial cells (ECs) seem to be a good target for gene and cell therapy. Moreover, ECs express major histocompatibility complex class II (MHC II) and they are considered semiprofessional antigen-presenting cells, so they may mediate the immune response that rises in around 30% of patients (Merlin et al. 2017a, Shi et al. 2020).

b. Adeno-associated vectors

AAVs are small, non-enveloped icosahedral viruses belonging to the Parvovirus family. AAVs have the ability to infect both dividing and non-dividing cells, but since they remain episomal, they are better suited for gene replacement therapy in non-dividing cell therapies. However, it has been observed that they can occasionally integrate into the host genome. A recent study of gene therapy in HA dogs using AAV examined the integration sites in liver samples from six treated dogs and revealed 1,741 distinct AAV integration occurrences in genomic DNA and approximately 44% of these integrations were found in proximity to genes associated with cell growth (Nguyen et al. 2021). These findings endorse the clinical advancement of liver-targeted AAV gene therapy for HA, underscoring the necessity for monitoring to assess potential genotoxic effects in the long term.

However, this is not the only limitation of AAVs. Other problems include widespread pre-existing immunity against the most common serotypes (Mingozzi and High 2013) and a limited

capacity for transgene size, accommodating small DNA inserts up to a maximum of 4.5kb (in contrast to lentiviral vector which can contain up to 10 kb) (Grieger and Samulski 2012). To address several of these challenges, the FVIII molecule has undergone significant modification, including the removal of the B-domain, which is not essential for clotting function, and codon optimization of the coding sequence. These modifications have enabled a reduction in the size of the FVIII cDNA, bridging the gap with the limited capacity of the AAV cassette. However, due to their prevalence as non-pathogenic viruses that infect humans from childhood, pre-existing antibodies against AAV are often found in the general population. For this reason, individuals with inhibitors are currently excluded from this therapeutic approach.

Despite these limitations, some strategies for gene therapy for HA utilizing distinct AAV capsids have been explored and recent clinical trials are ongoing (Table 2) (Zangi et al. 2023). Valoctocogene roxaparvovec (trade name Roctavian) stands as the first approved gene therapy treatment for HA (Ozelo et al. 2022). However, the critical aspect of these studies will be reached during longer follow-up periods, demonstrating whether FVIII levels will remain stable or if there will be a progressive decline leading to complete loss of expression.

Drug identification	Gene delivery system (encodes)	Study stage	Status
Giroctocogene-fitelparvovec	AAVV2/6 (rFVIII cDNA)	Phase 3	Active, not recruiting
NCT03001830	AAV2/8 (FVIII-V3 cDNA)	Phase 1	Recruiting
NCT03876301	AAVV (BDD-FVIII cDNA)	Phase 3 is not started yet	–
PF-07055480	rAAVV2/6 ((BDD-FVIII cDNA)	Phase 2/3	Active, not recruiting
BAY2599023	AAVV hu37(BDD-FVIII cDNA)	Phase 1/2	Recruiting
ASC618	AAVV (BDD- codon-optimized FVIII cDNA)		Not yet recruiting
BAX 888	AAVV8 (BDD-FVIII cDNA)		Active, not recruiting

Table 2. Current clinical trials employing AAV vector gene therapy for HA. The table summarizes the clinical trials with AAV that are currently active or proposed for HA, their gene delivery system and the stage of the study. AAV, adeno-associated vector; HA, hemophilia A. (Zangi et al. 2023)

c. Lentiviral vectors

Lentiviruses belong to the Retroviridae family and are characterized by their single-stranded RNA genome, which can be retrotranscribed into a stable complementary DNA (cDNA) integrated into the host cell genome. Their integration capacity has made lentiviral vectors (LVs) the preferred choice for gene therapy aiming at permanent gene transfer, despite their tendency

to integrate into the genome randomly (Montini et al. 2006, Biffi et al. 2011). LVs possess an active transport mechanism for translocating genomic material into the nucleus after reverse transcription of the genomic RNA, independent of the cell cycle status. Indeed, they can transduce both non-dividing and dividing cells compared to γ-retroviral vectors which can integrate the reverse transcribed genome only in non-dividing cells (Korin and Zack 1998). LVs offer an additional advantage over retroviral vectors as they tend to integrate preferentially into the coding regions of genes. In contrast, retroviral vectors have the tendency to integrate into the 5′-untranslated region of genes (Wu and Dunbar 2011). Additional advantages of LVs include the absence of coding sequences for viral proteins and the capacity to accommodate expression cassettes of up to 7 Kb. Over the years, modifications were made to the lentivirus genome to construct safe vectors, achieved by segregating the cis-acting sequences and trans-acting genes into separate plasmid constructs (Dull et al. 1998, Follenzi et al. 2000). The most updated LVs consist in the third generation vectors which ensures higher safety by dividing necessary sequences and genes for viral production among four separate plasmids.

A possible limitation of LVs is the specificity: LVs are pantropic and can infect theoretically all kinds of cell types because are hybrid LVs with the Glycoprotein G of the vesicular stomatitis virus envelope. There are some ways to overcome this problem but two of them are the most used. Firstly, tissue-specific promoters can be used to selectively direct vector expression to the desired tissue or cell type. For instance, promoters specific to hepatocytes have been extensively utilized to achieve prolonged expression in the liver while reducing immune responses (Follenzi et al. 2004). Another recent study of our group demonstrated that specific targeting of FVIII in *in vivo* FVIII-producing cells (mainly LSECs) under its physiological promoter (pF8) leads to a stable expression and a reduced immune response (Merlin et al. 2019b). Indeed, delivering these LVs via tail vein injection in HA mice resulted in sustained production of therapeutic levels of FVIII, surpassing those achieved by specifically targeting ECs with the vascular endothelial cadherin (VEC) promoter. Notably, the pF8 exhibited activity in an organ-dependent manner, facilitating transgene expression in LSECs and splenic hematopoietic cells. Furthermore, no inhibitors were observed and stable tolerance was obtained (Merlin et al. 2019a).

Inducible promoters offer another versatile option in gene therapy, providing an additional layer of control over gene expression. These promoters can be activated or suppressed in response to specific physiological or external stimuli, allowing precise temporal and spatial regulation of

gene expression. Additionally, another level of cell targeting is achieved through post-transcriptional regulation mediated by microRNAs (miRNAs). Introducing complementary sequences to a specific miRNA target sequence (miRT) downstream of the expression cassette provides the opportunity to selectively decrease transgene synthesis in cell types expressing the chosen miRNA. For instance, to enhance the specificity of FVIII expression, our group incorporated miRT142.3p, miRT-126, and miRT-122, employed to suppress FVIII expression in hematopoietic cells, ECs, and hepatocytes, respectively. Consequently, FVIII expression was confined to either endothelial or myeloid cells using LVs containing endothelial-specific (LV-VEC) or myeloid-specific (LV-CD11b) promoters. Notably, employing the VEC promoter for transcriptional targeting in ECs, along with the inclusion or exclusion of miRT122-142, resulted in sustained therapeutic levels of FVIII expression in HA mice and a reduced immune response (Merlin et al. 2017b).

There are some ongoing or approved clinical trials involving the use of LVs, but the number is relatively low compared to those utilizing AAVs (Dunbar et al. 2018). Indeed, LVs for HA have not entered clinical practice yet, despite their extensive use in preclinical models. Recently, data on non-human HA primates showed LVs as a promising therapeutic approach (Milani et al. 2022).

d. CRISPR/Cas9 genome editing approach

In recent years, there has been significant advancement in genome editing technologies, which are based on engineered or bacterial nucleases. Unlike viral vectors, which are primarily involved in facilitating gene addition, genome editing approaches provide a precise tool for a wide range of genetic manipulations, including gene addition, gene ablation, and gene correction. The versatility and high precision of genome editing, coupled with its applicability across various settings, are useful aspects of this technology. Indeed, genome editing can be performed *ex vivo*, where cells are edited outside the body before being reintroduced, or *in vivo*, where the editing machinery is delivered directly into the body to effect changes at the genetic level within the organism. This flexibility opens new avenues for personalized medicine approaches according to individual patients' genetic profiles. Despite these promising advancements, the translation of genome editing technologies into clinical practice is still in its early stages compared to viral gene addition therapies (Dunbar et al. 2018).

Starting from 1987, researchers delved into investigating clustered regularly interspaced short palindromic repeats (CRISPRs) and associated Cas proteins in bacterial genome (Ishino et al. 1987). These CRISPR arrays were noted to contain short DNA sequences matching those in viruses, suggesting their role as adaptive immunity systems against viral infections. Research unveiled how CRISPR systems utilize RNA molecules transcribed from these arrays to guide Cas proteins, allowing them to cut and eliminate viral genome (Barrangou et al. 2007). The CRISPR-Cas9 protein complex, in conjunction with its associated guide RNA (gRNA), stands as the most commonly employed genome editor.

Among all applications of this system, it is worth remembering the possibility to knock in or knock out a specific gene with high precision. The gRNA guides Cas9 to the target site, inducing a double-stranded DNA break repaired by cellular pathways such as nonhomologous end joining (NHEJ), which typically leads to a knockout gene introducing random insertion or deletion, or the more precise homology-directed repair (HDR) pathway utilizing a repair template that can be exploited to knock in a specific gene (Figure 6) (Wang and Doudna 2023).

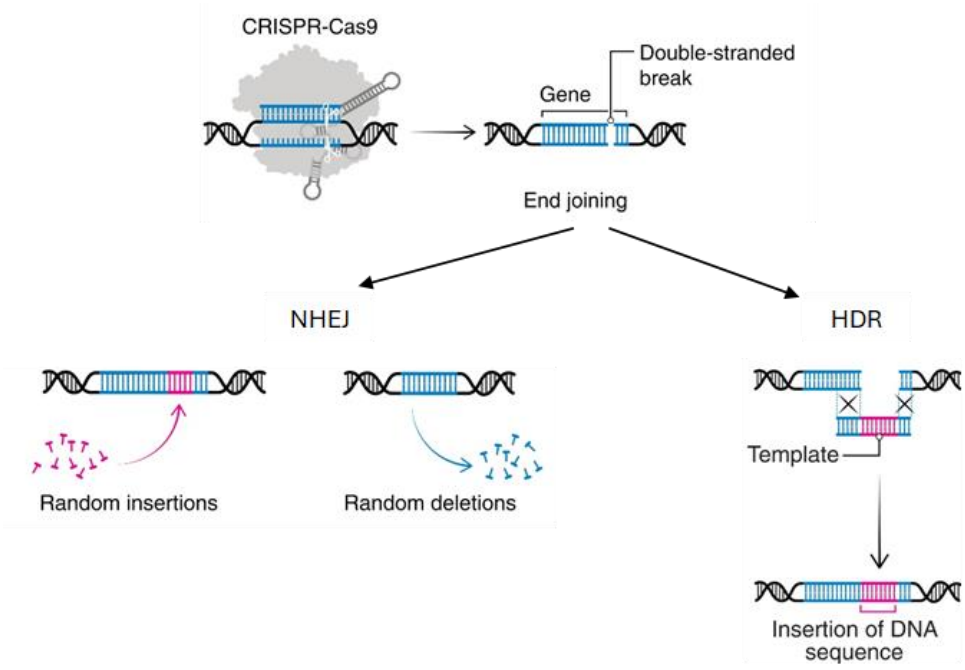


Figure 6. CRISPR-Cas9 genome editing. Guide RNA bring Cas to the site of interest where Cas cuts DNA obtaining a double-stranded break. This break can be repaired NHEJ or by HDR using a DNA template. These events bring to gene knockout or knock in respectively. CRISPR, clustered regularly interspaced short palindromic repeats; NHEJ, nonhomologous end joining; HDR, homology-directed repair. Adapted from (Wang and Doudna 2023)

Other applications of this gene editing tool consist of the speed of creating knockout mice, genetic screens, multiple genetic modifications, and site-specific modification using base editing (Wang and Doudna 2023). Moreover, it has been developed a system which takes advantage of an inactive or dead Cas9 to control gene expression by the regulation of the transcriptional machinery or the epigenetic regulators (Qi et al. 2013). Overall, CRISPR-Cas discovery led to precise alteration of DNA sequences in various cells, revolutionizing genetic manipulation techniques and affecting genetic disease treatment and agricultural practices (Wang and Doudna 2023).

Importantly, CRISPR/Cas9 technology has been used in recent years to correct genetic mutations in HA *in vitro* and *in vivo* systems. For instance, Int22Inv was corrected in induced pluripotent stem cells (iPSCs) from HA patients, leading to the expression of the *F8* in differentiated endothelial cells in HA mice (Park et al. 2015). Additionally, it has been demonstrated that corrected patient-derived iPSCs could be differentiated into $CD157^{+}$ cells, endothelial cells-like, which upon transplantation in HA mice effectively controlled bleeding episodes (Son et al. 2022). Furthermore, CRISPR/Cas9-mediated HDR has been employed to insert the functional FVIII gene into patient-derived iPSCs, demonstrating restored FVIII expression and activity (Sung et al. 2019). Interestingly, this method has recently achieved *in vivo* genetic correction in a model of HA. Researchers created an HA mouse model by removing the promoter region and exon 1 of *F8*, then, they packaged donor DNA and CRISPR/SaCas9 into AAV vectors and administered them intravenously to HA mice. Following the treatment, FVIII expression was restored, and the activated partial thromboplastin time (aPTT) was reduced (Luo et al. 2021). These results pave the way for future *in vivo* gene repair applications to potentially treat mutations in HA patients.

4. Clinical manifestations of hemophilic A patients

HA is a disabling condition characterized by chronic pain, joint dysfunction, and diminished quality of life that can begin early in life. Generalized bleeding is recognized as a hallmark of HA, occurring in various areas of the body, with joints and muscles being the most prominently affected sites (Gooding et al. 2021). Frequency and severity of spontaneous bleeding episodes

change according to the FVIII levels in the plasma. HA is classified as severe form, in which the levels of FVIII are lower than 1%, moderate form, between 1 and 5%, and mild form from 5 to 40% of FVIII activity (Bolton-Maggs and Pasi 2003). Severe HA is marked by more frequent occurrences of spontaneous bleedings, resulting in progressive damage primarily at the joint level. The onset of the disease occurs at early age in these patients while bleeding episodes occur only after trauma or surgery in patients with mild and moderate HA (Franchini et al. 2010, Hay et al. 2021).

Beyond bleeding, other pathological conditions seem to be correlated with the lack of FVIII as arthropathy, bone remodeling, renal and cardiovascular diseases and intracranial hemorrhages. Moreover, recent clinical and pre-clinical evidences have revealed that HA patients may suffer from vascular problems. However, it is not yet understood if these clinical complications are caused only by prolonged sedentary behavior of these patients and repeated hemorrhages or if the absence of the FVIII protein can be responsible for these clinical events.

a. Main clinical events in hemophilic A patients and pre-clinical models

Hemarthrosis and arthropathy

Joint bleeding, or hemarthrosis, is a hallmark of HA, predominantly affecting knees, ankles, and elbows (Luck et al. 2004, Lobet et al. 2014). It occurs due to the lack of functional FVIII, which impairs clot formation and leads to prolonged bleeding into the joint space after trauma or spontaneously. The high vascularization of synovial joints makes them susceptible to spontaneous bleeds, with the synovium as primary site for these events. When bleeding persists, it overwhelms the synovial clearance mechanism, leading to an accumulation of blood and, consequently, to an inflammatory status of the joints which stimulates neoangiogenesis (Roosendaal and Lafeber 2003, Leuci and Dargaud 2023). Hemarthrosis manifests with symptoms such as joint pain and reduced range of motion. Repeated bleeding episodes into the joints can cause chronic synovitis and subsequent joint damage, leading to arthropathy characterized by cartilage and bone deterioration and joint malformations.

While a comprehensive understanding of all molecular mechanisms underlying arthropathy remains elusive, there are indications suggesting certain involved pathways. First, it has been demonstrated in HA mice that blood-induced arthropathy activates nuclear factor (NF)-κB

pathway and increased expression of factors associated with hypoxia, angiogenesis, and chondrocyte damage within the affected joints (Sen et al. 2013). Second, in the same HA mice with arthropathy, angiogenic and hypoxia markers were found increased and their physiological expression was restored upon reintroduction of a specific microRNA (miR-15b) (Sen and Jayandharan 2016). Finally, it has been recently demonstrated the possible role of long non-coding RNA in the development of hemophilic arthropathy (Sarangi et al. 2023). Overall, these findings shed light on the possible molecular mechanisms that cause arthropathy and could have implications for the development of new therapies for joint diseases.

In this context, prophylactic treatment plays a pivotal role in managing HA, especially in preventing hemarthrosis. By reducing recurrent joint bleeding, prophylaxis helps preserve joint function, mobility, and quality of life in individuals with HA. Additionally, early initiation of prophylactic treatment has been shown to be effective in minimizing hemarthrosis and its long-term consequences.

Bone remodeling

Another typical clinical manifestation of HA patients is a reduction in bone mineral density, commonly observed across both pediatric and adult populations, coupled with an elevated fracture risk (Gay et al. 2015, Ulivieri et al. 2018, Gebetsberger et al. 2022). Initially, this decrease in bone mineral density was attributed to sedentary habits and recurrent joint bleeding. However, prophylactic approaches have led to safer hemostatic management, resulting in a lasting shift in patients' lifestyles. Nonetheless, while these replacement therapies partially maintain bone mineral density, they do not fully restore it (Khawaji et al. 2009, Alito et al. 2023).

Thus, it has been hypothesized that FVIII could play a role in bone remodeling but the interaction between FVIII with osteoblasts and osteoclasts remains partially controversial. In fact, it was shown that FVIII-vWF complex binds to osteoprotegerin and RANK ligand regulating bone remodeling (Lin et al. 2023), but the molecular mechanism of this function has not been unveiled yet. Furthermore, the process underlying bone turnover in hemarthrosis is believed to be controlled by RANK-L/RANK/OPG pathway (Melchiorre et al. 2012, Gebetsberger et al. 2022). Indeed, HA patients with severe arthropathy, synovial tissue exhibits

elevated levels of RANK and RANK-L, promoting osteoclast differentiation and subsequent bone resorption (Baud'huin et al. 2009).

Cardiovascular disease and inflammation

HA patients show lower cardiovascular mortality rates compared with the general population (Van Der Valk et al. 2022), exhibiting, however, similar prevalence or even more common rates of cardiovascular risk factors such as hypertension (Fransen van de Putte et al. 2012, Von Drygalski et al. 2013, Barnes et al. 2016). Additionally, they also have equivalent levels of atherosclerosis compared with general population (Kamphuisen and Ten Cate 2014, Badescu et al. 2023). This phenomenon raises questions about the mechanisms underlying cardiovascular outcomes in HA patients. From one side, the reduced cardiovascular mortality observed in HA patients might be attributed to decreased thrombus formation due to their hypocoagulable status. From the other side, the same hypocoagulable status can be the cause of the lower the stability of atherosclerotic plaques increasing possible thrombitic events in these patients. More clinical and pre-clinical data are needed to have a full comprehension of the cardiovascular risks of HA patients. Moreover, considering the altered pressure status of these patients, it is not a surprise that these individuals also present higher renal disease compared with the general population (Quon and Konkle 2010, Esposito et al. 2013).

Finally, a general pro-inflammatory environment has been assessed in HA patients showing increased levels of cytokines correlated to inflammation, especially interleukine-6, neutrophil activation and bone turnover (Czajkowska et al. 2022, Noone et al. 2023). Importantly, the assessment of bone turnover and NETs markers could help in the identification of patients with an increased risk of developing hemophilic arthropathy and abnormalities in bone metabolic turnover. These results were corroborated by another study which demonstrated that young HA patients show a pro-inflammatory status and a higher number of microparticles derived from derived from endothelium, erythrocytes, platelets, leucocytes, neutrophils, and T lymphocytes before being treated with FVIII (Jardim et al. 2017). Overall, these data suggest that HA patients show a different immunological profile which can affect also the physiology of other cells such as endothelial ones.

b. Intracranial hemorrhages

Intracranial hemorrhages (ICHs) affect around 3-5% of newborns with severe HA during the perinatal period. It is noteworthy that ICHs can also occur in adults, resulting in disability and, in severe instances, even mortality, with an approximate fatality rate of 20% (Stieltjes et al. 2005, Ljung 2008, Zanon et al. 2012, Zanon and Pasca 2019, Zwagemaker et al. 2021). Hypertension and cardiovascular diseases are the primary risk factors for ICHs in HA adult patients (Von Drygalski et al. 2013). Therefore, intracranial microbleeds have been demonstrated to be more frequent in HA patients compared with the general population (Husseinzadeh et al. 2018).

The onset of ICHs is often spontaneous, and the exact underlying cause remains uncertain. Although the precise mechanisms leading to ICH development are still unclear, it appears to be a correlation between the frequency of ICHs, the severity of the hemophilia condition, and the age of the patients (Bladen et al. 2016, Andersson et al. 2017). Importantly, the implementation of prophylactic treatment has emerged as a pivotal strategy in mitigating the risk of ICHs in HA patients (Aras and Oral 2020, Zanon et al. 2022a). Indeed, pediatric patients receiving prophylaxis demonstrate a reduced risk of ICHs in contrast to those children utilizing non-frequent or no prophylaxis (Andersson et al. 2017). The varying clinical outcomes observed with replacement or non-replacement therapy remain an unresolved issue which needs further investigation (Albattat et al. 2023, Yamada et al. 2023).

Finally, another aspect worth exploring is the variation in the occurrence and severity of ICHs between HA and HB patients. Notably, many studies failed to differentiate between these two patient groups, likely due to the limited availability of recruitable HB patients. These investigations could help determine whether the absence of FVIII (or FIX) plays a direct role in these bleeding incidents or if they arise from the hypocoagulable state characteristic of both forms of hemophilia.

c. Endothelial dysfunction and extracellular matrix impairment

Some studies demonstrated a possible general endothelial dysfunction in HA patients highlighted by a decreased flow-mediated dilation (FMD) and reduced hyperemic velocity time integral (VTI) when compared to healthy controls, pointing to significant alterations in both

macrovascular and microvascular endothelial functions (Sartori et al. 2008, Biere-Rafi et al. 2012, Sun et al. 2017). These data were corroborated by a recent study in which miRNA-155, miRNA-1, and miRNA-197 were found significantly elevated in HA patients compared to controls, correlating strongly with increased interleukin-6 (IL-6) and soluble intercellular adhesion molecule-1 (ICAM-1) levels, markers of endothelial dysfunction (Böhmert et al. 2019, Noone et al. 2023). Also pro-angiogenic markers have been identified increased in synovium and peripheral blood of HA patients with joint disease underscoring the possible altered endothelial functionality in these patients (Acharya et al. 2011). Similarly, it has been demonstrated elevated angiogenesis in the synovial membrane of patients with hemophilic arthropathy, coupled with heightened VEGF expression at the joint level (Zetterberg et al. 2014). Certainly, alterations in the blood levels of certain substances produced by ECs serve as recognized indicators of endothelial damage and dysfunction (Mućka et al. 2022).

Therefore, several groups have reported the relation between collagen quantity and patients' annual bleeding rate. For instance, high levels of plasma collagen XVIII have been observed in HA patients and they correlate to high annual bleeding rates (Kjeld et al. 2018a). Collagen XVIII has been also demonstrated to be a potential marker for monitoring the development of hemophilic arthropathy (Tantawy et al. 2022). Moreover, recently, markers of type IV collagen turnover (PRO-C4 and C4M), and of type VIII collagen synthesis (PRO-C8) have been shown to be elevated in the plasma of HA patients (Manon-Jensen et al. 2024a). These evidences corroborate the possible endothelial dysfunction of these patients and indicate an impairment of the extracellular matrix (ECM) metabolism. Furthermore, these data also correlate with abnormal synovial perfusion, thin vessel wall formation and joint bleeding.

Finally, studies involving FVIII-deficient mice have revealed substantial alterations in joint vascular remodeling following hemarthrosis induction (Cooke et al. 2018). This suggests an uncontrolled, non-physiological angiogenic response that may exacerbate prolonged and recurrent bleeding episodes. This concept is further supported by observations of increased synovial vascular permeability post-joint injury in these mice (Bhat et al. 2015, Cooke et al. 2018, 2019, Gopal et al. 2021), underscoring the vulnerability of the vascular system in the absence of FVIII.

Overall, these evidences underscore the significance of endothelial dysfunction and a possible impaired ECM turnover in HA patients. Thus, it can be supposed that bleeding episodes originate not only from clotting deficiencies but also from vascular irregularities.

5. Interplay between coagulation factors and endothelial cells

Coagulation factors (CFs) play a pivotal role in hemostasis, participating in the intricate process of blood clot formation. They are categorized into pro- and anti-CFs based on their respective functions in promoting or blocking coagulation. They orchestrate a finely tuned cascade of enzymatic reactions to ensure the timely and effective formation of blood clots when needed. Anti-CFs act as regulatory checkpoints, counterbalancing the pro-CFs to prevent uncontrolled clotting and maintain vascular integrity. Through intricate interactions with platelets, ECs, and various plasma proteins, CFs actively participate in the dynamic regulation of clot formation (Flier et al. 1992, Madhusudhan et al. 2015).

Beyond hemostasis, angiogenesis is an essential process for vessel repair. It involves vascular sprouting and the formation of new blood vessels, necessary for wound closure. Upon vascular injury, initial vessel constriction occurs to control blood flow and minimize hemorrhage, followed by the exposure of the sub-endothelial matrix for platelet adhesion. Vascular endothelial cells play a pivotal role by initially binding and anchoring the clot, then invading the fibrin structure to form a new vessel wall from the clot margins (Browder et al. 2000). This intricate process is finely regulated by numerous proteins released by both blood and ECs, ensuring proper repair and healing of the injured vessels. Among all these proteins, recent findings have demonstrated that CFs affect EC functions, playing an essential role beyond hemostasis. Indeed, they have been shown to contribute significantly to the intricate modulation of angiogenic processes and the regulation of EC barrier function (Olgasi et al. 2024).

Delving into the intricate interplay between hemostasis and angiogenesis presents a promising avenue for unraveling the complexities of vascular dynamics. By analyzing the intersection of these two physiological processes, researchers can gain deeper insights into the mechanisms governing vascular repair and remodeling. Such investigations hold significant potential for

advancing our knowledge of vascular biology and may ultimately lead to the development of novel therapeutic strategies for various vascular-related disorders.

a. Pro- and anti-coagulation factors in endothelial cell functionality

Among all the CFs, some of them have been studied in depth in the control of EC functions, especially thrombin, tissue factor (TF) among pro-CFs and antithrombin (AT), activated protein C (APC), and thrombomodulin (TM) as representatives of anti-CF class (Olgasi et al. 2024). Thrombin is a pivotal enzyme in hemostasis cleaving fibrinogen into fibrin and it also triggers inflammation and signaling pathways via G protein-coupled receptors (GPCRs) like PAR1, impacting various cell types, including ECs, where it increases permeability by disrupting the endothelial barrier (Rabiet et al. 1996). Indeed, thrombin's binding to GPCRs regulates Ca^{2+} levels and cytoskeleton rearrangement in ECs, enhancing permeability (Hirano and Hirano 2022). Furthermore, it has been demonstrated a role of thrombin in regulating angiogenic process. Firstly, it has been described to increase angiogenic factors enhancing tumor development (Catar et al. 2021). Additionally, thrombin induces new vessel formation post ICHs (Zhou et al. 2012) and regulates vascular development during murine embryonic (Griffin et al. 2001). Importantly, its binding with integrin αvβ3 promotes EC attachment, migration, and survival (Tsopanoglou et al. 2002).

Similarly, TF is involved both in coagulation and angiogenesis. During the coagulative process, it binds activated factor VII (aFVII) inducing the activation of FX and leading to fibrin deposition. In addition, it has been demonstrated a direct role on EC homeostasis though integrins α6β1 and αvβ3 or RAR2 regulating migration and tubulogenesis (Van Den Berg et al. 2009, Zhu et al. 2011). Both *in vitro* and *in vivo* data show its essential role in the development and maintenance of correct vasculature (Unruh and Horbinski 2020).

Conversely, one of the major anti-CFs, AT, which inhibits thrombin, FX, and FIX, has been found as a potent anti-angiogenic factor downregulating the expression of genes related to extracellular matrix assembly in ECs (Zhang et al. 2006). It has also been demonstrated to control inflammation, proliferation, migration, and capillary-like tube formation binding heparan sulfate proteoglycans (HSPGs) on EC surface (Panicker et al. 2020). A protective effect on EC barrier stability has been described for APC through the binding with angiopoietin-1 receptor (Tie2) or PAR1 (Soh and Trejo 2011, Minhas et al. 2017), while controversial results

have been reported about the role of TM in the regulation of angiogenesis (Kuo et al. 2012, Hsu et al. 2016). The known binding partners of the discussed CFs are reported in Figure 7.

Importantly, it is essential to recognize the significance of other factors like FV and factor XIII (FXIII), which have been identified to promote EC migration and maintain vascular homeostasis (Cui et al. 1996, Dardik et al. 2006). Moreover, FIX has been implicated in regulating EC permeability (Mamiya et al. 2016) and factor I (FI), FVII, and factor XII (FXII) have been shown to have pro-angiogenic properties (Reinhardt et al. 2015). Unfortunately, the angiogenic potential of FX remains debated, with uncertainty regarding whether it acts in a pro- or anti-angiogenic capacity (Reinhardt et al. 2015). These findings underscore the dual role of CFs as key participants in both coagulation and angiogenesis.

In this scenario, the potential impact of FVIII on EC homeostasis has been explored, although limited information exists regarding its specific role in EC function. As already mentioned, the clinical manifestations of HA patients suggest that the absence of FVIII could be responsible for uncontrolled and impaired vascular homeostasis. Furthermore, recent investigations have been delving into the relationship between FVIII and EC functionality in *in vitro* models. Specifically, it has been demonstrated that FVIII can influence the attachment and the permeability of human umbilical vein endothelial cells (HUVECs) modifying their transcriptomic profile (Cadé et al. 2022b). However, this study shows some limitations that need to be elucidated. First, HUVECs are not the ideal model to study FVIII (Shovlin et al. 2010a). Second, the molecular mechanism triggered by FVIII in ECs has been not fully described. Finally, the authors speculate that lipoprotein receptor related protein 1 (LRP-1) could be the binding partner of FVIII that induces an intracellular signaling within the cells but they do not provide any evidence. Many receptors control the bioavailability of FVIII including LRP-1, low density lipoprotein receptor, stabilin-2, C-type lectin domain family 4 member M, asialoglycoprotein receptor and scavenger receptor class A member 5, but none of them have been described to transduce a signaling related to EC functionality after FVIII binding (Figure 7) (Cadé et al. 2022a). Further research needs to be conducted to determine if and how FVIII interacts with ECs.

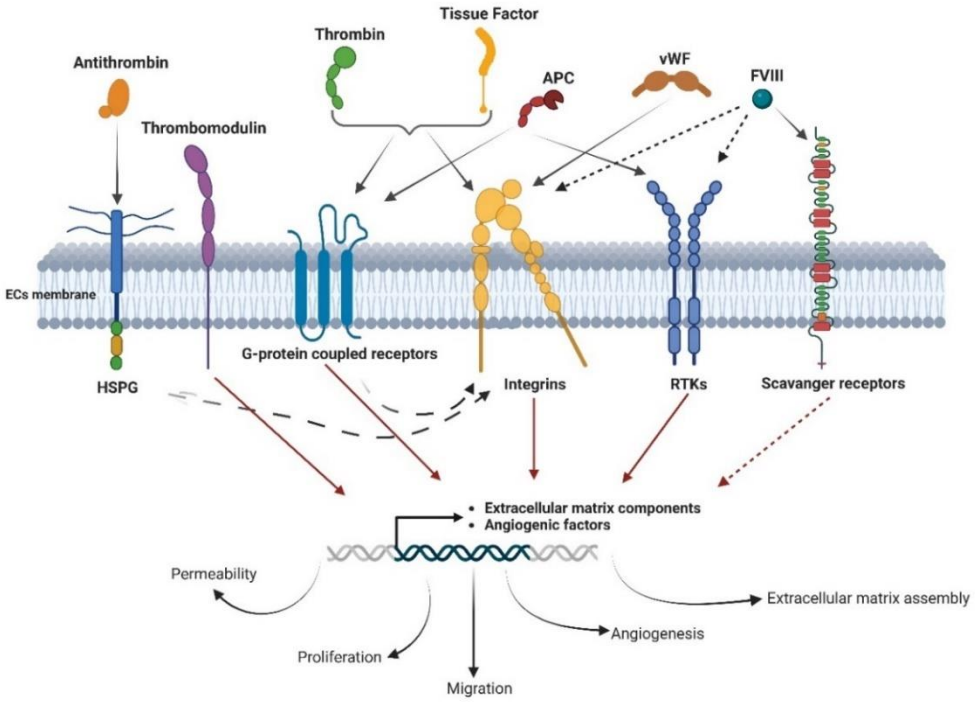


Figure 7. Coagulation factors interacting with receptors implicated in endothelial cell functions. AT binds to HSPGs. TM expression can induce the expression of genes involved in extracellular matrix and angiogenesis. Thrombin and TF bind GPCRs and integrins. APC binds GPCRs and RTKs. FVIII has been observed to interact with established scavenger receptors, yet its potential engagement with integrins or RTKs in regulating EC functionality remains largely unexplored. Activation of these receptors has been shown to influence the expression of genes related to extracellular matrix organization and angiogenesis, thereby modulating EC functions. AT, antithrombin; HSPGs, heparan sulfate proteoglycans; TF, tissue factor; APC, activated protein C; GPCRs, G-protein coupled receptors; RTKs, receptor tyrosine kinases; EC, endothelial cell. (Olgasi et al. 2024)

b. Extracellular matrix organization and integrins in endothelial cells

Key endothelial processes such as cell adhesion, migration, proliferation, and, especially, angiogenesis are regulated through changes in the ECM (Marchand et al. 2019, Saio et al. 2021). Moreover, ECM ensures that the endothelial barrier remains intact and responsive to physiological needs, thereby playing a pivotal role in vascular homeostasis.

The ECM is a complex network of proteins and polysaccharides that provides structural and biochemical support to surrounding cells. It can actively influence cell behavior through biochemical and mechanical signals, playing a crucial role in tissue development, differentiation, and homeostasis (Dufort et al. 2011). The composition of the ECM varies throughout the body to facilitate appropriate cell-cell interactions based on the specific function

of the local tissue. The specialized ECM that surrounds and supports ECs is called basement membrane, and it is primarily composed of collagen IV, laminin, nidogen, and the heparan sulfate proteoglycan perlecan (Pollard et al. 2016, Marchand et al. 2019). Collagen IV and laminin are the most abundant elements and their network is stabilized by perlecan and nidogen, especially nidogen 2, found to be the most abundant nidogen in the basement membrane (Kohfeldt et al. 1998, Lummerstorfer 2001, Mutgan et al. 2020) However, how nidogen helps in the assembly and stabilization of the basement membrane is still a theme of discussion and need to be elucidated (Töpfer and Holz 2024).

The relation between ECs and ECM components is reciprocal: from one side, ECs express ECM proteins to regulate the surrounding environment; from the other side, the elements of the ECM modulate the functions and the phenotype of these cells.

ECM-EC interaction is mainly allowed by integrins (Post et al. 2019). Indeed, these transmembrane receptors have been described to regulate angiogenesis and wound healing in ECs mediating interactions with the ECM (Hynes 1992, Avraamides et al. 2008, Mezu-Ndubuisi and Maheshwari 2020, Yu et al. 2024). Integrin involvement in these processes ensures proper vascular formation and repair, highlighting their importance in maintaining physiological EC function and promoting tissue regeneration. Beyond their classical role, these receptors have been deeply described to bind non-ECM ligands and regulate many physiological functions (Lafoya et al. 2018). Integrins also act as cell-surface receptors for several growth factors, hormones, small molecules, and CFs (as described above) triggering specific intracellular pathways and, thus, playing a crucial role in modulating EC behavior (Lafoya et al. 2018, Aman and Margadant 2023, Olgasi et al. 2024).

Signaling pathways activated by the binding between integrins and various ligands may lead to the phosphorylation of several proteins and, consequently, to downstream changes in the composition and organization of the ECM (Ivaska and Heino 2000). Among these intermediate intracellular actors activated through integrins, the most important is focal adhesion kinase (FAK). Once activated, the FAK molecules undergo cross-phosphorylation on a specific tyrosine residue, forming a phosphotyrosine docking site that attracts members of the Src family, subsequently leading to the amplification of the signal within the cell (Mitra and Schlaepfer 2006). The importance of FAK in ECs was demonstrated by knocking it out in mice

ECs which led to early embryonic death, caused by vascular instability, and impaired angiogenesis (Shen et al. 2005, Braren et al. 2006).

To summarize, we could say that ECM and various circulating molecules can bind integrins which transmit an outside-in signal mainly through FAK leading to a new organization of ECM itself and, thus, to a profound regulation of pivotal EC functions (e.g. angiogenesis, migration and permeability). As mentioned above, CFs emerge as potential integrin-binding partners triggering intracellular signaling in ECs and modulating their profiles (Figure 7) (Olgasi et al. 2024). All these key evidences have paved the way for future researches to unveil the intricate relationship between CF-integrins-ECM.

c. Blood outgrowth endothelial cells as optimal model to study hemophilia A

Most of the studies mentioned above were conducted in *in vitro* endothelial cells which mainly consist of immortalized cell lines or primary HUVECs. These models are widely used in research because are easy to obtain and culture. Specifically, immortalized cell line such as EA.hy926 can be maintained for more than 100 population doublings (ATCC). On the other hand, HUVECs can be isolated from the human umbilical vein through an enzymatic digestion (Jaffe et al. 1973, Hewett 2016) and maintained in culture up to 5/6 passages. One significant drawback of HUVECs is their inability to be isolated from individual patients, preventing them from accurately replicating the genetic profile of a specific pathology without genetic manipulation. Foreskin-derived human dermal microvascular ECs (HDMECs) or human dermal blood microvascular ECs (HDBECs) serve as viable alternatives, but they consist of mixed populations of blood and lymphatic endothelial cells (Petrova et al. 2002, Nowak-Sliwinska et al. 2018). Other primary ECs can be isolated from endothelial biopsies and, thus, they maintain the same mutations of the patient, but they are challenging to recover and possess a limited capacity for proliferation (Hewett 2016). iPSCs have been demonstrated to be useful to generate patient-specific ECs, lowering the risk of immune rejection and providing an autologous cell source for potential transplantation. A limitation of these cells consists in the difficulty to obtain desired ECs expressing mature markers.

For these reasons, BOECs represent an appealing option for model patient-specific ECs. BOECs constitute a subset of human endothelial cells which can be isolated from the peripheral blood.

Lin et al. were the first to document the existence of circulating cells capable of differentiating into cells displaying the characteristic traits of vascular endothelium (Lin et al. 2000). Studies dating back to 1997 have investigated the concept of isolating endothelial cells from blood, identifying circulating progenitor cells for the endothelial lineage (Asahara et al. 1997). However, subsequent research clarified that these endothelial progenitor cells (EPCs) lacked the ability to undergo a stable lineage switch to the endothelium. Instead, they comprised various hematopoietic cells capable of serving paracrine pro-angiogenic functions for vascular repair and replacement but unable to fully integrate as ECs in injured vasculature (Medina et al. 2017a). Later studies revealed that these cells are, in fact, mononuclear cells, and the endothelial markers were acquired thanks to the fusion with platelet microparticles (Prokopi et al. 2009). Many controversial studies were published about the identity and the origin of BOECs until Hebbel finely described the differences between 3 kinds of blood endothelial cells (Figure 8) (Hebbel 2017).

Circulating ECs (CECs) emerge from the detachment of endothelial cells from the vessel wall, typically in response to injuries or pathological circumstances. While CECs exhibit a restricted capacity for proliferation, they offer valuable insights into the status of the endothelium, serving as indicators of vascular health and dysfunction (Solovey et al. 1997). Conversely, by culturing peripheral blood mononuclear cells, it is possible to obtain cells that can be classified in EPCs or BOECs. The cells referred as EPCs have a hematopoietic origin, and they are characterized by limited growth potential and inability to maintain a stable and mature endothelial phenotype (Yoder et al. 2007, Medina et al. 2017b). In contrast, BOECs originate from *in vitro* culture and exhibit complete EC differentiation across various parameters including morphology, phenotype, organelle content, responsiveness to stimuli, observable behaviors, and gene expression patterns (Hebbel 2017). These cells are derived from a lineage of circulating, marrow-derived, transplantable cells, often referred to as endothelial progenitor cells or endothelial colony-forming cells (ECFCs) (Lin et al. 2000). For classification as ECFCs, these cells must exhibit positive cell surface markers associated with endothelial characteristics such as CD31, CD144, CD146, vWF, and VEGFR-2, along with negative cell surface marker expressions for leukocytic markers including CD45, CD14, and CD68 (Hebbel 2017, de Boer et al. 2020). Despite their predominant residence in the vessel wall, a sufficient number of ECFCs circulate in the blood, and they are detected in the early stages of cultures from which

BOECs eventually develop (Yoder et al. 2007). This classification has been recently confirmed by another group with slight differences, specifically EPCs have been called myeloid angiogenic cells (MACs) to define both their lineage and function (Ng and Cheung 2024).

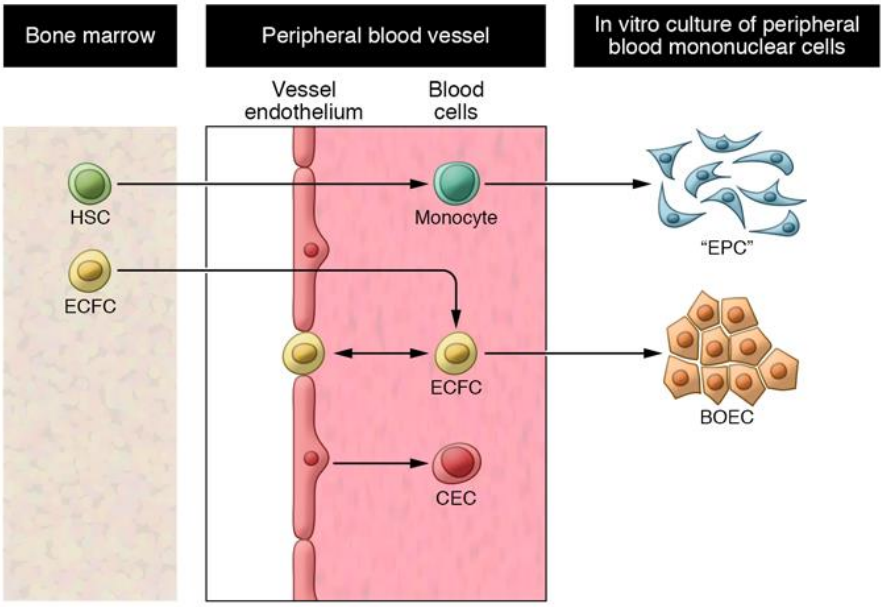


Figure 8. Different kinds of blood endothelial cells, their origin and culture. CECs are detached from the vessel wall endothelium as the result of injury. BOECs are mature endothelial cells and derived from ECFCs, a marrow-derived progenitors present in both circulating blood and within the endothelium. EPCs show a hematopoietic stem cell origin and can be cultured *in vitro*. CECs, circulating endothelial cells; BOECs, blood outgrowth endothelial cells; ECFCs, endothelial colony–forming cells; EPCs, endothelial progenitor cells. (Hebbel 2017)

Nowadays, BOECs are widely used because of their several advantages, particularly when sourced from diseased individuals, they have the potential to recapitulate various genetic, biochemical, and phenotypic aspects of the underlying vascular pathophysiology. This ability to reflect the specific conditions of patients makes BOECs valuable tools for studying disease mechanisms and developing targeted therapies. Overall, the use of BOECs enhances our understanding of vascular diseases and facilitates the development of more effective therapeutic strategies. Therefore, BOECs have captured significant interest due to their potential in translational studies and gene therapy, also for HA (Paschalaki and Randi 2018).

Indeed, BOECs have been used to delivery FVIII in immunocompromised mice demonstrating that these cells are a valuable tool for gene and cell therapy sustaining therapeutic levels of

FVIII up to 5 months (Lin et al. 2002). Similar data were obtained when LV-FVIII transduced BOECs were implanted in Matrigel plugs in mice maintaining therapeutic levels of FVIII up to 27 weeks (Matsui et al. 2007). To improve these results in longer-term and higher expression of FVIII, a cell-sheet technology was implemented to obtain a monolayer of cells transplantable subcutaneously (Tatsumi et al. 2013). Also, autologous LV-FVIII BOECs have been implanted into the omentum of normal and HA dogs where FVIII was detected in circulation up to one year, indicating extended cell survival and FVIII production (Ozelo et al. 2014). Moreover, to achieve stable, long-term engraftment, BOECs were combined with placenta-derived mesenchymal stromal cells (PMSCs) and transplanted in murine neonates (Gao et al. 2019).

In this context, our group isolated BOECs from both healthy donors and HA patients exhibiting no discernible differences in terms of morphology and marker expression. LV-FVIII healthy and HA BOECs were expanded under GMP-compliant conditions showing no phenotypical and functional changes and, then, encapsulated into a pre-vascularized device (CellPouch™) already in phase III clinical trial for type I diabetes. This device facilitated the survival of BOECs and subsequent secretion of FVIII, reaching therapeutic levels of plasma FVIII for up to 13 weeks. This preclinical study confirmed the safety and feasibility of transplanting BOECs within an implantable device for HA (Olgasi et al. 2021).

Although BOECs cannot be isolated from the peripheral blood of all individuals due to low number of circulating ECFCs in the blood, standardized protocols and guidelines to successfully isolate and identify these cells have been recently published (Smadja et al. 2019). Nevertheless, BOECs stand as a potent tool for investigating molecular endothelial dysfunction in disease, providing access to ECs from both patients and healthy controls in a non-invasive manner.

Aim of the thesis

Hemophilia A (HA) is a hereditary bleeding disorder characterized by reduced or absent activity of coagulation factor VIII (FVIII). The severity of HA varies based on residual FVIII activity, with more frequent spontaneous bleeding episodes occurring in severe cases, often manifesting as hemarthroses and spontaneous intracranial hemorrhage (ICHs). Furthermore, studies have indicated potential endothelial dysfunction in HA patients, as evidenced by decreased flow-mediated dilation (FMD) and reduced hyperemic velocity time integral (VTI) compared to healthy individuals, suggesting alterations in both macrovascular and microvascular endothelial functions. Recently, elevated plasma levels of markers associated with various collagen turnover have been observed in HA patients, further supporting the notion of endothelial dysfunction and indicating potential impairment of extracellular matrix metabolism. Therefore, while various coagulation factors (CFs) are recognized for their roles in regulating endothelial cell (EC) functionality, the specific influence of FVIII on EC homeostasis is largely understudied.

Thus, the main objectives of my PhD thesis were: i) to characterize the role of FVIII in EC functions and homeostasis through *in vitro* and *in vivo* assays; ii) to investigate if FVIII could contribute to the physiological transcriptomic and proteomic profile of ECs regulating specific gene and protein expression inducing intracellular signaling in ECs; iii) to evaluate possible differences in the brain microvasculature of WT and HA mice, which could be the possible causes of ICHs in HA patients.

Overall, the aim of this work was to define new roles of FVIII in EC functionality highlighting its possible binding partner/s and defining if FVIII triggers an outside-in pathway in ECs. Finally, we focused on brain HA ECs to understand if the absence of FVIII may compromise the brain vasculature.

Gaining insights into how FVIII influences EC functionality can guide the advancement of novel therapeutic strategies, leading to improved management of HA, and may contribute to a higher comprehension of EC biology and vessel physiopathology.

Materials and Methods

Cell culture

HEK293T cells were cultured in Iscove's Modified Dulbecco's medium (IMDM) (Gibco®, Life Technologies) supplemented with 10% fetal bovine serum (Gibco®, Life Technologies) and 1% Penicillin-Streptomycin (100U/ml Euroclone) under humidified atmosphere of 5% CO_2 at 37 °C.

Human dermal microvascular endothelial cell line (HMEC-1) (ATCC, CRL-3243) were cultured in MCDB131 medium (Gibco®, Life Technologies) supplemented with 10% fetal bovine serum (Gibco®, Life Technologies), 10 ng/ml human Epidermal Growth Factor (Immunotools), 1 µg/ml hydrocortisone, 1% Glutamine (Euroclone) and 1% Penicillin-Streptomycin (100U/ml Euroclone).

BOECs were isolated from four healthy donors and four severe HA patients as previously described (Olgasi et al. 2021). They were maintained on CellCoat Collagen Type 1-coated Tissue Culture Flasks (Greiner Bio-One) at a 10^4 cells/cm^2 in MCDB 131 medium (Gibco®, Life Technologies) containing proprietary supplements. For all the functional assays, BOECs were used between passage 4 and 8.

In vitro tubulogenic assay

Twenty-four-well tissue culture plates were coated with 300 µl Matrigel® Matrix (Corning) per well and allowed to solidify at 37°C for 30 minutes. $5x10^4$ BOECs or $5x10^4$ HMEC-1, were resuspended in serum-free medium and placed on top of the Matrigel®. Plates were incubated at 37°C, 5% CO_2 and analyzed after overnight (ON) incubation. Cells were incubated on top of Matrigel® ON with 1 or 5 IU/ml of rhFVIII or with 1 µM defactinib (VS-6063, Selleck Chemicals), or the combination of both. Images were acquired under inverted microscope Leica ICC50. ImageJ Angiogenesis software of ImageJ was used to quantify the number of nodes, junctions, branches, and total length.

In vitro migration assay

BOECs or HMEC-1 were plated into the upper compartment of 8μm pore size Transwell (Corning) at a density of 10^5 cells in serum-free medium, while the lower compartment was filled with complete medium with 10% FBS and incubated ON. Cells were incubated on top of Matrigel® ON with 1 or 5 IU/ml of rhFVIII or 1 μM defactinib or a combination of both. After incubation, migrated cells were fixed with 70% ethanol and stained with 0.1% crystal violet (Sigma-Aldrich). The migrated cells were photographed under an inverted microscope Leica ICC50. The crystal violet was eluted with 10% acetic acid and quantified using Victor Spectrophotometer (PerkinElmer) at 590 nm.

In vitro permeability assay

Permeability was measured across a monolayer of BOECs or HMEC-1. Cells ($8x10^4$ cells/well) were plated on 0.1% gelatin coated transwell (3μm pore, 24-well format) and cultured until confluence was reached. Cells were incubated on top of the transwell with 1 or 5 IU/ml of rhFVIII or 1 μM defactinib alone or the combination of both added every 2 days. At the end of the culture, 50 μl (5 μg/ml) of FITC-conjugated 40-kDa dextran (Sigma-Aldrich) was added to the upper chamber and the fluorescence of the lower chamber was measured in the medium after 30 min of incubation using Victor Spectrophotometer at 490 nm (excitation)/520 nm (emission). Fluorescence readings were normalized to dextran permeability in transwell inserts without cells.

7-AAD staining for analysis of DNA content

C, HA and LV-FVIII HA-BOECs were analyzed to evaluate their DNA content and, thus, their proliferation status. Cells were detached and resuspended in PBS with 2mM EDTA. After the centrifuge, the supernatant was removed and 70% ice cold EtOH was added dropwise while vortexing. This procedure allows the permeabilization of the cells that were maintained for 1h at 4°C. Cells were washed with FACS buffer (PBS, 2%FBS, 2mM EDTA) and treated 30min with 100 ug RNAse at 37 °C (Thermoblock). To stop the reaction, the cells were transferred in ice and 1 ug of 7-AAD (7-amino-actinomycin D) was added for 30min before the analysis. We evaluated the fluorescence of 7-AAD by FACS (488nM excitation 647nM emission).

Proliferation assay with Incucyte

C and HA BOECs or WT and HA BMECs were plated at a density of 20.000 cells/cm2 in serum-free medium. Proliferation assay was performed with or without 1UI/ml of rhFVIII for BOECs. The cells were maintained in the Incucyte® for 48 hours and every 4 hours a photo was taken. Confluence analysis was calculated by artificial intelligence integrated software and the proliferation rate was normalized at time 0.

Guide RNA design and cloning

In collaboration with Paola Capasso, gRNAs against the human *F8* gene were designed using the website tool CHOP CHOP (https://chopchop.cbu.uib.no). Three guides showing high activity score and no predicted off-target activity were selected for both exon 4 and 7 (Table 3). The CHOP CHOP tool was used to design primers to amplify the regions encompassing the Cas9-cleavage sites. The gRNA sequences (Table 4) were ordered as oligo by Sigma Aldrich, with appropriate overhangs, annealed and cloned by BbsI digestion (New England BioLabs) into a LV transfer construct containing the U6 gRNA-expression cassette into the self-inactivating Long Terminal Repeats (LTRs) and encoding for the marker gene GFP from the human PGK promoter (pCCLsin.cPPT.hPGK.eGFP.Wpre.3'LTR-U6.gRNA loxP or LV-Cas9). Concerning the cloning of the Cas9-expressing LV, it was generated by replacing the Cas9 promoter of the pCW-Cas9 plasmid (Addgene No. 50661) with the SK-T6 promoter41.

gRNA selection in HEK293T cells

The LV plasmids coding for the gRNAs were individually transfected with a Cas9-expressing plasmid in HEK293T cells by electroporation using the 4D-Nucleofector™ System (Lonza, Basel, Switzerland) following the manufacturer's instructions. Briefly, HEK293T were cultured to 60–70% confluence, then harvested and washed with phosphate buffered saline. Approximately 5×10^5 cells were resuspended in the nucleofection solution SF together with 1µg of the Cas9 plasmid and 500 ng of the gRNA-expressing plasmid and transfected using instrument's program CM-130. 10 days post-nucleofection, genomic DNA from transfected cells was extracted using Maxwell 16 LEV Blood DNA kit (Promega) and the region

encompassing the Cas9 cleavage sites in exon 4 or 7 were PCR amplified using primers listed in Table 5 and according to this conditions: 30 cycles of heating at 95 °C for 30 s, 58 °C for 30s, and 72°C for 1 min, with a final extension of 2 min at 72°C amplify. PCR amplicons were then subjected to the T7 endonuclease mismatch assay (New England Biolabs) according to the manufacturer's instruction. The T7-treated PCR products were resolved using High Resolution Precast Electrophoresis Gel Spreadex® (AL-Diagnostic-GMBH) and visualized by staining with Atlas ClearSight Dna Stain (BioAtlas) using the Gel Doc System (Biorad). Cas9-induced mutations at exon 4 and 7 was quantified and, based on these data, the two guides, which resulted in the higher editing efficiency, were chosen for further experiments.

CRISPR/Cas9 two-vector system

C-BOECs and HMEC-1 cells were LV transduced with a two vectors CRISPR/Cas9 system (Follenzi and Naldini 2002, Amendola et al. 2013). The first vector contains *S. pyogenes* Cas9 nucleotide sequence under the control of a doxycycline synthetic promoter and the puromycin resistance gene under the control of human PGK promoter (Figure 9). Transduced cells were selected with a puromycin (1 ug/ml; Sigma) enriched medium prior the second LV transduction. The second vector carries the gRNA sequence against *F8* under the control of U6 promoter and the GFP under the control of human PGK promoter was used as a second selection marker (Figure 9). Two gRNAs against the *F8* were chosen amongst the six designed, Ex4 gRNA 3 and Ex7 gRNA1 (Olgasi et al. 2023). Cas9 expression was induced with Doxycycline (Sigma) at 75 ng/ml for 72 h.

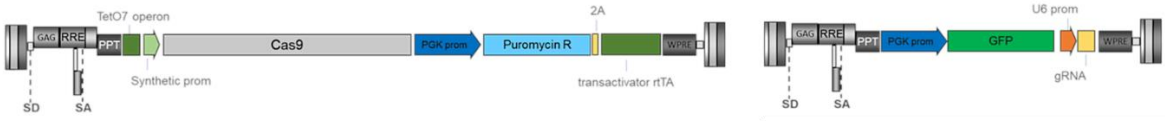


Figure 9. Plasmids of CRISPR/Cas9 two vector system
HMEC-1 and C-BOECs were transduced with a third generation LV carrying Cas9 under the control of a doxycycline inducible synthetic promoter and the puromycin resistance gene under the control of PGK promoter (left). A second LV construct, carrying the gRNA sequence under the control of U6 promoter and the GFP under the control of PGK promoter, was used to transduce ECs + Cas9 (right).

FVIII Activity and FVIII Antigen Assay

To assess FVIII activity, aPTT assay was performed on supernatants of cultured cells. Standard curves were generated by serial dilution of recombinant human FVIII (rhFVIII) in culture medium. Samples and standards were diluted 1:5 in FVIII-deficient plasma (TECO). All the experiments were performed using a Coatron M4 coagulometer (TECO Medical Instruments) and TEClot APTT-S kit reagents (TECO Medical Instruments). FVIII antigen in supernatant of cultured cells was quantified by ELISA sandwich using the Matched-Pair Antibody Set for ELISA of human FVIII antigen (Affinity Biologicals) following the manufacturer's protocol. For standard curve generation, known concentrations of commercial rhFVIII were serially diluted in culture medium.

RNA-seq

RNA from BOECs was purified as previously described (Incarnato et al. 2014), and its integrity measured using Fragment Analyzer™ (Advanced Analytical). Library preparation was performed from PolyaAplus RNA using Illumina TruSeq RNA prep-kit as previously described. Samples were run in the Illumina sequencer NextSeq 500.

Sequencing reads were aligned to human reference genome (version GRCh38.p13) using STAR v2.7.7a0 (Dobin et al. 2013) (with parameters –outFilterMismatchNmax 999 –outFilterMismatchNoverLmax 0.04) and providing a list of known splice sites extracted from GENCODE comprehensive annotation (version 32) (Frankish et al. 2019). Gene expression levels were quantified with feature Counts v1.6.3 (Liao et al. 2014) (options: -t exon -g gene_name) using GENCODE gene annotation (version 32 basic). Multi-mapped reads were excluded from quantification. Gene expression counts were next analyzed using the edgeR package (Robinson et al. 2010). Normalization factors were calculated using the trimmed-mean of M-values (TMM) method (implemented in the calcNormFactors function) and RPKM were obtained using normalized library sizes and gene lengths. After filtering the lower expressed genes, a differential expression analysis was carried out by fitting a GLM to all groups and performing QL F-test for the interesting pairwise contrasts, blocking on patients when possible. Genes were considered as significantly differentially expressed (DEGs) when having |logFC|

>1 and raw p value < 0.01 in each reported comparison, as advised by SEQC consortium (Su et al. 2014).

After the identification of a dataset of differentially expressed genes (DEGs), Enrichr online tool was used to identify pathways and gene ontology (GO) terms enriched using DEGs as input. A term is defined as significantly enriched if the reported adjusted p value is < 0.01.

Gene set enrichment analysis (GSEA) was performed using a Broad Institute java package version 3.0 (classic mode) and MSigDB.

Proteomic analysis

Cells were collected, washed, lysed with RIPA buffer and sonicated. Proteins were then precipitated with cold acetone and resuspended. Proteins were then reduced in 25 µL of 100 mM NH_4HCO_3 with 2.5 µL of 200 mM DTT (Merck) at 60°C for 45 minutes and next alkylated with 10 µL 200 mM iodoacetamide (Merck) for 1 hour at RT in dark conditions. Iodoacetamide excess was removed by the addition of 200 mM DTT. The digests were dried by Speed Vacuum and then desalted (Manfredi et al. 2016).

Digested peptides were analyzed on an Ultimate 3000 RSLC nano coupled directly to an Orbitrap Exploris 480 with a High-Field Asymmetric Waveform Ion Mobility Spectrometry System (FAIMSpro) (all Thermo Fisher Scientific). Samples were injected onto a reversed-phase C18 column (15 cm × 75 µm i.d., Thermo Fisher Scientific) and eluted with a gradient of 6% to 95% mobile phase B over 80 min by applying a flow rate of 300 nL/min, followed by an equilibration with 6% mobile phase B for 8 min. MS scans were performed in the range of m/z 375–1200 at a resolution of 120,000 (at m/z = 200). MS/MS scans were performed choosing a resolution of 15,000; normalized collision energy of 30%; isolation window of 2 m/z; and dynamic exclusion of 45 s. Two different FAIMS compensation voltages were applied (−45 V and −60 V), with a cycle time of 1.5 s per voltage. FAIMS was operated in standard resolution mode with a static carrier gas flow of 4.6 L/min.

The acquired raw MS data files were processed and analyzed using Proteome Discoverer with Chimerys (v3.0.0.757, Thermo Fisher Scientific). SequestHT was used as a search engine and the following parameters were chosen. Database: Homo sapiens (Uniprot, downloaded on 01-02-2018) enzyme: trypsin; max. missed cleavage sites: 2; static modifications: carbamidomethyl (C); dynamic modifications: oxidation (M); precursor mass tolerance: 10

ppm; fragment mass tolerance: 0.02 Da. Only peptides and proteins with FDR value < 0.01 were reported. Abundance of identified peptides was determined by label-free quantification (LFQ) using match between runs. Statistical analyses and t-test were performed on protein abundances using MetaboAnalyst software (https://www.metaboanalyst.ca/). Proteins were considered as significantly differentially expressed when having |FC| >1 and p value < 0.05. Modulated proteins were analyzed through Database for Annotation, Visualization and Integrated Discovery (DAVID) (version 6.8) (http://david.abcc.ncifcrf.gov/). Cluster analysis was performed with STRING.

Intact Cell Respiration Using High-Resolution Respirometry

Cellular respiration was determined using an Oroboros oxygraph-2K high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria) and the "substrate, uncoupler, inhibitor, titration" (SUIT) protocol SUIT-003_O2_ce_D012 recommended by the manufacturer of the Oroboros instrument as previously described (Raiteri et al. 2021). BOECs were detached from the flask using trypsin-EDTA (Gibco), counted using a hemocytometer, and resuspended in pre-warmed respiration medium MiR05 (0.5 mM EGTA, 3.0 mM MgCl2·6H2O, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, 1 g/L bovine serum albumin, pH 7.1) to achieve a final cell density of $1x10^6$ cells/mL. A couple of BOECs from C, HA and LV-FVIII HA were analyzed simultaneously in the neighboring chambers and oxygen concentration and flux were recorded using DatLab software (Oroboros). Baseline oxygen consumption rates were identified for each chamber during the "Routine" phase without and with pyruvate (5 mM) stimulation, used to sustain TCA-linked respiration in MiR05 medium. Subsequently, oligomycin (5 nM) was added to inhibit ATP synthase and assess uncoupled respiration ("Leak" phase). The protonophore carbonyl cyanide p-(trifluoro- methoxy) phenylhydrazone (FCCP) was then titrated (0.05 µM increments) until peak oxygen flux was achieved, indicative of maximal respiration ("Electron transport (ET)" phase). Finally, 1 µl each of rotenone (0.5 µM) and antimycin A (2.5 µM) were sequentially added to inhibit ETC complexes I and III, respectively, and identify ET-independent respiration (ROX phase). Rates of O_2 consumption (flux) were normalized to total protein content. Briefly, at the end of the experimental procedure, the cellular suspension from the two chambers was centrifuged at 1000 g for 5min. The cellular pellet was lysed in 200 µL of lysis buffer (10 mM

HEPES, 60 mM KCl, 1 mM EDTA, 0.075% NP40, 1 mM DTT) and then centrifuged at 15,000 × g for 15 min at 4 °C. The concentration of the protein in the supernatant was measured with Bradford Reagent.

Flow-TriCEPS

To investigate the binding of FVIII to cell surface receptor(s), ligand-based receptor capturing was applied on HA-BOECs by using Flow-TriCEPS (Dualsysteums Biotech AG, Schlieren, Switzerland). This assay was performed according to the manufacturer's instructions. Briefly, rhFVIII was conjugated to one of the three arms of the Flow-TriCEPS system and the same procedure was performed for the positive and the negative control, transferrin and glycine respectively. Then, living BOECs were incubated at different conditions of temperature and pH with these systems. Samples are subsequently incubated with streptavidin-fluorophore (PE) and analyzed using flow cytometry. Binding of the ligand to cell surface receptor(s) leads to higher fluorescence intensity compared to the negative control.

FVIII immunoprecipitation

A total of 10 µg of sheep anti-human FVIII antibody (Affinity Biologicals) was combined with 50 µl of Protein G Dynabeads (ThermoFisher) and incubated for 2 h at RT in 300 µl PBS, 0.02% Tween 20 (PBS-T) with a gentle rotation to ensure antibody binding to the beads. Following washing, a total of 150 µg of whole cell lysate in a volume of 300 µl containing 1xHALT protease inhibitors (ThermoFisher), was then incubated with the antibody-beads complex ON at 4°C with a gentle rotation. Successively, the supernatant was collected (POST IP) to ensure protein quality, while the bead IP complex was washed a total of 5 times in PBS-T. After that, the beads were resuspended in 25 µl of PBS and analyzed in combination with the POST IP by immunoblotting under denaturing conditions for the presence of integrin β1 (1:4000; Cell Signalling) or FVIII (1:1000; Green Mountain).

Western Blot

HA-BOECs were cultured in spent medium for 96 h. 1 U/ ml of rhFVIII or 50 ng/mL of VEGF-A was added for 15 min at 37 °C and whole cell lysates were prepared using RIPA buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 1× protease inhibitor cocktail from Sigma-Aldrich) with concentrations determined using the Pierce BCA Protein Assay (Thermo Fisher Scientific). For time-course analysis, HA-BOECs were stimulated for 48 h with 3 U/ml of rhFVIII by adding rhFVIII daily. The samples were size-fractionated on 8% SDS-PAGE under reducing conditions and electro-transferred to immuno-blot polyvinylidene difluoride membrane (BioRad). Membranes were incubated with the following antibodies: anti-NID2 (1:3000, Invitrogen); anti-Vinculin antibody (1:10000, SantaCruz); anti-phospho-FAK Tyr 397 (1:3000, Cell Signaling); anti-FAK (1:3000, Cells Signaling); anti-phospho-Src Tyr 416 (1:1000, Cell Signaling); anti-Src (1:1000 Santa Cruz); anti-phospho AKT Thr 308 (1:1000, Cell Signaling); anti-AKT (1:1000, Sigma-Aldrich); anti-phospho-mTOR Ser 2448 (1:500; Cell Signaling); anti-mTOR (1:1000 Sigma-Aldrich); anti-phospho-p38 (1:1000, Cell Signaling); anti-p38 (1:4000, Cell Signaling); anti-phospho-MAP Kinase 1/2 (Erk1/2)(Thr185/Tyr187) (1:2000, Sigma-Aldrich); Anti-MAPK 1/2 (ERK1/2) (1:3000, Sigma-Aldrich); anti-phospho-VEGF Receptor 2 (Tyr1175) (1:2000, Invitrogen); anti-phospho-Tyrosine (P-Tyr-100) (1:2000, Cell Signaling); anti-Tubulin (1:3000, Sigma-Aldrich) (Table 6). Blots were visualized with the appropriate horseradish peroxidase-conjugated secondary antibody. Immunoreactive proteins were detected using enhanced chemiluminescence (Clarity Western ECL Substrate; Bio Rad) with image capture performed using ChemiDoc Touch Imaging System (BioRad).

Animal procedures

NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ, Jackson #005557 (NSG) mice were purchased by Charles River, while the NSG-HA mice were previously generated and maintained in our laboratory (Zanolini et al. 2015b). We also used WT and HA C57Bl/6 from Charles River Spa. HA mice were obtained from WT adding a cassette of neomycin after exon 16 of *F8* gene (Bi et al. 1995). All animals' procedures were performed under a sterile hood.

In vivo permeability assay

Evans blue extravasation was used to quantify the capillary permeability in 8 weeks old NSG and NSG-HA mice. For FVIII delivery, 4 IU/mice of rhFVIII were tail vein injected every 2 days for 20 days. NSG-HA mice were also tail vein injected with $5x10^8$ TU/ mouse of LV-FVIII. The FVIII activity was evaluated over time for up to 50 days. At the end of the experiment, a 0.5% Evans Blue solution (Sigma-Aldrich) was injected into the tail vein. After 15 min, mice were killed, and the extravasation was visualized in the interstitial space under the skin of the mice.

Isolation of brain microvascular endothelial cells (BMECs)

We optimized a protocol to obtain BMECs based on published protocols (Ruck et al. 2014, Assmann et al. 2017, Czupalla et al. 2018, Bernard-Patrzynski et al. 2019). Briefly, brains were harvested from WT or HA 8 weeks old C57Bl/6 mice. Meninges were detached and the tissue was minced with a pestle and, then, digested with collagenase II (1mg/mL) and DNAse (60 U/mL) for 1 h at 37 °C. To remove the myelin, the digested tissue was resuspended in DMEM 20% BSA and centrifuge 1000 g for 20 min 4 °C. The floating myelin was discarded and the pellet of cells was digested another time with dispase (0,8 U/mL) and DNAse (40 U/mL) for 1h 37 °C. The isolated cells were furtherly purified though a percoll® gradient 1000 g 10 min 4 °C. Isolated BMECs were recovered, analyzed by FACS for CD31 and CD45 and plated on collagen-coated plates in the same medium as BOECs. Both WT and HA BMECs were maintained in culture for 4 days. Immunostaining and proliferation were assessed.

Immunostaining

Murine brains were harvested from WT and HA C57Bl/6 mice and fixed in 4% PFA for 2 h at 4°C and embedded in cryostat embedding medium (Bio-Optica). Cryostat sections of 15 µm thickness were blocked in buffer containing 5% goat serum, 1% BSA, and 0.1% Triton X-100 in PBS, incubated with primary antibody followed by the secondary antibody at RT. For nuclei detection DAPI was added to the secondary antibodies' solution. Images of stained histological sections were acquired using Axioscan microscope (Zeiss) and were analyzed with Zeiss ZEN

3.7 software (Zeiss). Primary and secondary antibodies and dilutions are reported in Table 6. Vascular density was analyzed with Vessel Analysis plugin of ImageJ software.

To stain murine BMECs, the cells were cultured over glasses and, when confluent, fixed in 4% PFA for 2 h. They were blocked in buffer containing 5% goat serum, 1% BSA, and 0.1% Triton X-100 in PBS, incubated with primary antibody followed by the secondary antibody at RT. Images were acquired using Leica DM5500 microscope and analyzed with Leica application suite X software (LAS-X). Primary and secondary antibodies and dilutions are reported in Table 6.

shRNA plasmid generation

NID2 specific predesigned shRNA lentiviral plasmid was purchased from shRNA MISSION® (Sigma-Aldrich). ITGB1-specific shRNA oligonucleotides designed were cloned into pLKO.1 vector at AgeI and EcoRI restriction sites (shITGB1.1 TTTGTAGGAAGAGGGATAATA; shITGB1.2 GCCTTGCATTACTGCTGATAT). shRNA against scramble sequence (shScramble; negative control) was already available in our laboratory (SHC002, MISSION® Control Vectors, Sigma-Aldrich).

Lentiviral vector generation

Third generation self-inactivating LVs were produced as previously published (Follenzi and Naldini 2002). Briefly, HEK293T cells were expanded and transiently transfected by the calcium phosphate precipitation method with four plasmids encoding for two core packaging constructs (pMDLg/pol and pRSV-Rev), the envelope construct (pMD.VSV.G), and the transfer vector construct (pPGK.FVIII.2APuroRc; pPGK.PuroR; pPGK.NID2; pPGK.shScramble; pPGK.shNID2; pPGK.shITGB1.1; pPGK.shITGB1.2; pF8P.FVIII; pPGK.Cas9; pPGK.GFP.U6promoter.gRNAex4F8; pPGK.GFP.U6promoter.gRNAex7F8). The cell supernatant was harvested, and LV particles were concentrated by ultracentrifugation. The LVs expressing GFP were titrated by FACS, while the other LVs were quantified as number of integrated copies by quantitative-PCR (qPCR).

Cell transduction

HA-BOECs were plated at a 10^4 cells/cm^2 density and after 6–8 h transduced with a lentiviral vector carrying the BDD form of FVIII.2A.PuroR under the control of the ubiquitous PGK promoter, or a LV carrying the Nidogen2.2A.PuroR or puromycin resistance gene under the control of PGK promoter using a multiplicity of infection (MOI) of 20. HA-BOECs were LV transduced with LV.shITGB1.1 or LV.shITGB1.2 or LV.shScramble with a MOI of 10. C-BOECs and HMEC-1 were LV transduced with LV.shNID2 or LV.shScramble or LV.Cas9 or LV. gRNAex4F8 or LV. gRNAex7F8 with a MOI of 10.

Analysis of lentiviral vector copy number

Vector copy number on genomic DNA from LV-FVIII transduced BOECs was performed as previously described8. Briefly, SYBR green (Thermo Fisher Scientific) qPCR was carried out with the following primers: Forward TTGCTTCCCGTATGGCTTTC, Reverse AGCTGACAGGTGGTGGCAAT.

Flow cytometry analysis

C, HA and LV-FVIII HA-BOECs were analyzed for the following markers: CD31, CD144, KDR, CD34 and CD45 (Table 6). HMEC-1 and C-BOECs transduced with LV-gRNA were analyzed to assess the GFP expression. HA-BOECs transduced with LV-shITGB1 and their controls were analyzed to assess ITGB1 expression. The proliferation rate of C, HA and LV-FVIII HA BOECs was assessed by FACS, evaluation the fluorescence of 7AAD. Isolated BMECs were analyzed by FACS to detect their CD31 and CD45 expression (Table 6). For each sample, 1.5×10^5 live events were acquired on the Attune NxT Acoustic Focusing Cytometer (ThermoFisher Scientific, Waltham, MA, USA). Data were analyzed by FlowJo™ V10 Software (BD Bioscience).

Statistical analysis

All data were expressed as mean ± SD. Graphs were generated, and statistical analyses were performed using Prism 8 (Graph Pad). The p-values were calculated using t-test (when two groups were compared) or one-way ANOVA with a Bonferroni post-hoc test (when more than two groups were compared). In Figure 28, two-way ANOVA with a Bonferroni post-hoc test has been performed to verify the difference of the sample over time. $p < 0.05$ values were considered statistically significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Table 3. Sequences of the crRNA present in the gRNAs

Ex4 gRNA 1	GTACTAGTAGGGCTCCAATG
Ex4 gRNA 2	GGACCTGCCAGACATATGTA
Ex4 gRNA 3	GTGGAAGCCATACATATGTC
Ex7 gRNA 1	GCTTGTGAGGAACCATCGCC
Ex7 gRNA 2	GAACCATCGCCAGGCGTCCT
Ex7 gRNA 3	GTGCACTCAATATTCCTCGA

Table 4. Oligos for gRNA cloning

F8.Ex4_1f	ACCGTACTAGTAGGGCTCCAATG
F8.Ex4_1r	AAACCATTGGAGCCCTACTAGTA
F8.Ex4_2f	ACCGGACCTGCCAGACATATGTA
F8.Ex4_2r	AAACTACATATGTCTGGCAGGTC
F8.Ex4_3f	ACCGTGGAAGCCATACATATGTC
F8.Ex4_3r	AAACGACATATGTATGGCTTCCA
F8.Ex7_1f	ACCGCTTGTGAGGAACCATCGCC
F8.Ex7_1r	AAACGGCGATGGTTCCTCACAAG
F8.Ex7_2f	ACCGAACCATCGCCAGGCGTCCT
F8.Ex7_2r	AAACAGGACGCCTGGCGATGGTT
F8.Ex7_3f	ACCGTGCACTCAATATTCCTCGA
F8.Ex7_3r	AAACTCGAGGAATATTGAGTGCA

Table 5. Oligo for the amplification of exon 4 and 7 of F8

F8.Ex4.T7_F	TTGAGTGTACAGTGGATATAGAAAGG
F8.Ex4.T7_R	TCAGGTGAAGGAACACAAATGC
F8.Ex7.T7_F	TCATAGCCATAGGTGTCTTATTCC
F8.Ex7.T7_R	ATGTTGGTGGGAAGAGATATGAC

Table 6. Primary and secondary antibodies

Antigen	Reactivity	Manufacturer	Format	
CD31	human	clone MEM-05; Invitrogen	APC	1:50
KDR	human	clone ES8-20E6; Miltenyi Biotec	PE	1:100
CD144	human	Clone HI30; Invitrogen	PE	1:100
CD34	human	Clone 4H11; Immunotools	PE	1:200
CD45	human	Clone HI30; Invitrogen	APC	1:100
CD31	mouse	Clone MEC 13.3 BD Pharmingen™	APC	1:50
CD45	mouse	Clone 30-F11 Thermo Fisher	AF780	1:100
CD31	mouse	Clone Mec 13.3 Biolegend	Biotin	1:200
αSMA	mouse	Clone 1A4 Abcam	Not conjugated	1:100
CD45	mouse	Clone 390 eBioscience	Not conjugated	1:100
CLDN5	mouse	Polyclonal Thermo Fisher	Not conjugated	1:100
		Streptavidin eBioscience	APC	1:500
/	/	Streptavidin eBioscience	PE	1:500
NID2	human	Polyclonal Thermo Fisher	Not conjugated	1:3000

Vinculin	human	Clone H-10 Santa cruz	Not conjugated	1:10000
IgG (H + L)	mouse	Polyclonal Thermo Scientific	488	1:500
IgG (H + L)	rabbit	Polyclonal Thermo Scientific	546	1:500
IgG (H + L)	mouse	Polyclonal Thermo Scientific	HRP	1:5000
IgG (H + L)	rabbit	Polyclonal Thermo Scientific	HRP	1:5000
phospho FAK	human	Clone D20B1 Cell Signaling	Not conjugated	1:3000
FAK	human	Polyclonal Cell Signaling	Not conjugated	1:3000
phospho Src (Tyr 416)	human	Polyclonal Cell Signaling	Not conjugated	1:1000
Src	human	Polyclonal Santa Cruz	Not conjugated	1:1000
phospho AKT (Thr308)	human	Polyclonal Cell Signaling	Not conjugated	1:1000
AKT	human	Clone SKB1 Sigma-Aldrich	Not conjugated	1:1000
phospho mTOR (Ser 2448)	human	Clone D9C2 Cell Signaling	Not conjugated	1:1000
mTOR	human	Clone 21D8.2 Sigma-Aldrich	Not conjugated	1:500
phospho p38 (Thr180/Tyr182)	human	Clone D3F9 Cell Signaling	Not conjugated	1:1000
p38	human	Clone D3E1 Cell Signaling	Not conjugated	1:4000
ITGB1	human	Clone TS2/15 Invitrogen	FITC	1:100
ITGB1	human	Clone D6S1W Cell Signaling	Not conjugated	1:3000
FVIII	human	Polyclonal Affinity Biologicals	Not conjugated	1:100
FVIII	human	Clone GMA-012 Green Mountain	Not conjugated	1:1000

phospho VEGFR2 (Tyr1175)	human	Polyclonal Invitrogen	Not conjugated	1:2000
Phosphor Tyrosine (P-Tyr-100)	human	Clone PTyr100 Cell Signaling	Not conjugated	1:3000
phospho-MAP Kinase 1/2 (Erk1/2)(Thr185/Tyr187)	human	Clone AW39 Sigma-Aldrich	Not conjugated	1:2000
MAP Kinase 1/2 (ERK1/2)	human	Polyclonal Sigma-Aldrich	Not conjugated	1:2000
Tubulin	human	Clone DM1A Millipore	Not conjugated	1:3000

Results

FVIII is essential for human endothelial cell function

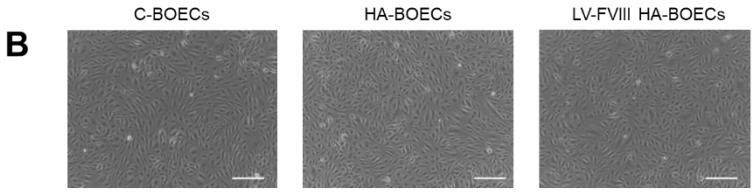
To investigate the function of FVIII in ECs, we isolated BOECs from healthy donors (C-BOECs) or severe HA patients (HA-BOECs) with different mutations (Figure 10A). BOECs showed the typical cobblestone-like morphology (Figure 10B) and expressed classical endothelial markers (i.e., CD31, CD144, KDR), while they were negative for the hematopoietic markers CD34 and CD45 (Figure 10C). We also transduced HA-BOECs with a LV carrying the *F8* transgene (LV-FVIII HA-BOECs) to further investigate the possible role of FVIII in ECs lacking FVIII (Figure 10B). Transduced cells harbored approximately five integrated LV copies/cell (Figure 10D) and secreted substantial levels of FVIII, as judged by the marked difference in FVIII secretion rates (83 ± 9 ng/10^6 cells for C-BOECs; 4 ± 0.3 ng/10^6 cells for HA-BOECs; and 390 ± 84 ng/10^6 cells for LV-FVIII HA-BOECs) (Figure 10E) and the shortening of activated partial thromboplastin time (aPTT) in cell supernatants (90 ±11 s for C-BOECs, 114 ± 13 s for HA-BOECs, 58 ± 14 s for LV-FVIII HA-BOECs) (Figure 10F).

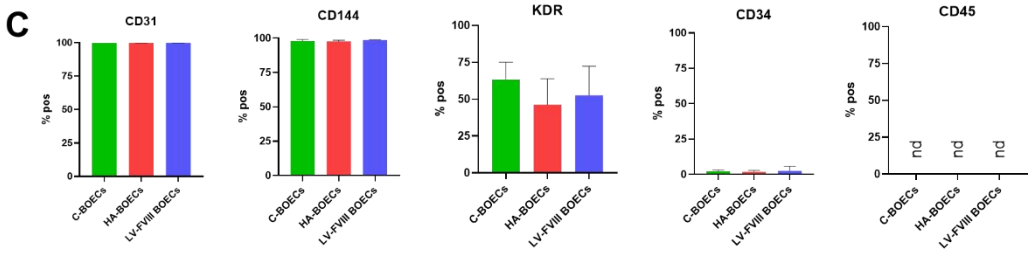
Importantly, we found that HA-BOECs formed a sparse and incomplete vascular network on Matrigel® in contrast to C-BOECs, which developed a complete and stable vascular network (Figure 11A, B). Their functional impairment was also observed in migration and permeability assays (Figure 11C, D). Importantly, LV-FVIII HA-BOECs showed a significantly improved tubule network formation, migration, and permeability, similar to the results seen in C-BOECs (Figure 11B-D). Thus, demonstrating that in addition to its pro-coagulative function, FVIII could play a role in EC functionality.

To determine whether treatment of BOECs with rhFVIII could increase their functionality, we challenged both C-BOECs and HA-BOECs with two different doses of rhFVIII (1 IU/ml and 5 IU/ml). Curiously, we observed that C-BOECs showed no further increase in functionality after rhFVIII treatment while HA-BOECs showed marked improvements in tubule formation, migration, and permeability (Figure 12A-D). These observed effects were independent to the rhFVIII dosage used as demonstrated by no significant differences in the outcomes between the two concentrations used. These findings collectively show that either ectopic expression of FVIII or *in vitro* rhFVIII administration are essential for EC functionality.

A

Name of HA patient	Degree of severity	Mutation
HA1	Severe HA	c.6273G>A
HA 2	Severe HA	IVS22
HA 3	Severe HA	unkown
HA 4	Severe HA	c.5881T>C





D

Sample	Copy number/cell
HA 1-BOECs non transduced	0
LV-FVIII HA 1-BOECs MOI 20	6
HA 2-BOECs non transduced	0
LV-FVIII HA 2-BOECs MOI 20	5
HA 3-BOECs non transduced	0
LV-FVIII HA 3-BOECs MOI 20	4
HA 4-BOECs non transduced	0
LV-FVIII HA 4-BOECs MOI 20	5

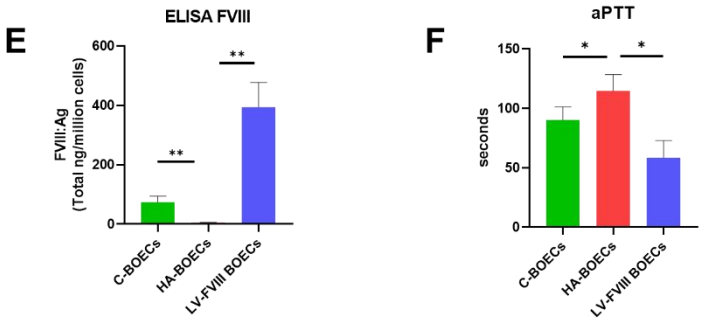


Figure 10. Characterization of healthy, HA and LV-FVIII HA-BOECs
A Table reporting the mutations in HA patients from whom the HA-BOECs were isolated. **B** Representative images of healthy control (C), HA and LV-FVIII HA-BOECs (n=4 for each group). Scale bar = 200 µm. **C** FACS histograms showing the expression of CD31, CD144, KDR, CD34, and CD45 in C, HA, and LV-FVIII HA-BOECs. **D** qPCR analysis of integrated LV copy number/cell in LV-FVIII HA-BOECs. **E** Antigen levels in supernatants from transduced and non-transduced of C, HA, and LV-FVIII HA-BOECs, as determined by ELISA, n=4 different biological samples for each group (two independent experiment have been performed for each biological sample). **F** aPTT assay results for the supernatants of C, HA, and LV-FVIII HA-BOECs, n=4 different biological samples for each group (two independent experiment have been performed for each biological sample). **C**, **E,** and **F** are expressed as mean ± SD. Statistical analysis was performed by t-test (**p < 0.01; *p < 0.05).

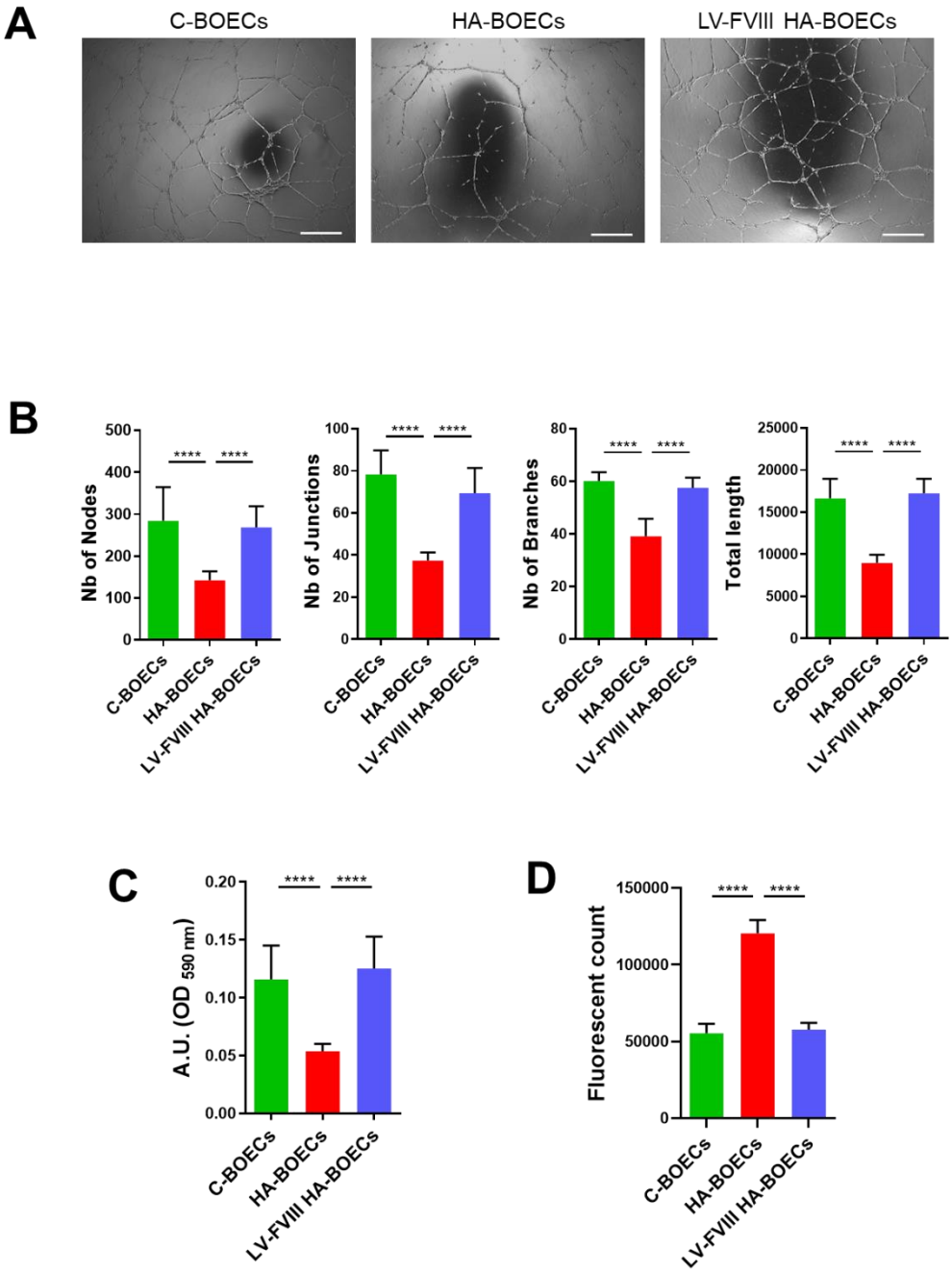


Figure 11. HA-BOECs are defective in tubulogenesis, migration and permeability.
A Representative images from a tubulogenic assay using C-BOECs (n=4), HA-BOECs (n=4), or LV-FVIII HA-BOECs (n=4). Scale bar = 500 µm. **B** Quantification of the number of nodes, junctions, branches, and total length of tubule networks. **C** Indirect measurement of cell migration by crystal violet staining elution. **D** Permeability assay results calculated from the extravasation of FITC-dextran through an intact monolayer. Data in **B**, **C**, and **D** are expressed as mean ± SD. All experiments were performed three times for each subject (4 for each group). Statistical analysis was performed by one-way ANOVA test (****$p < 0.0001$).

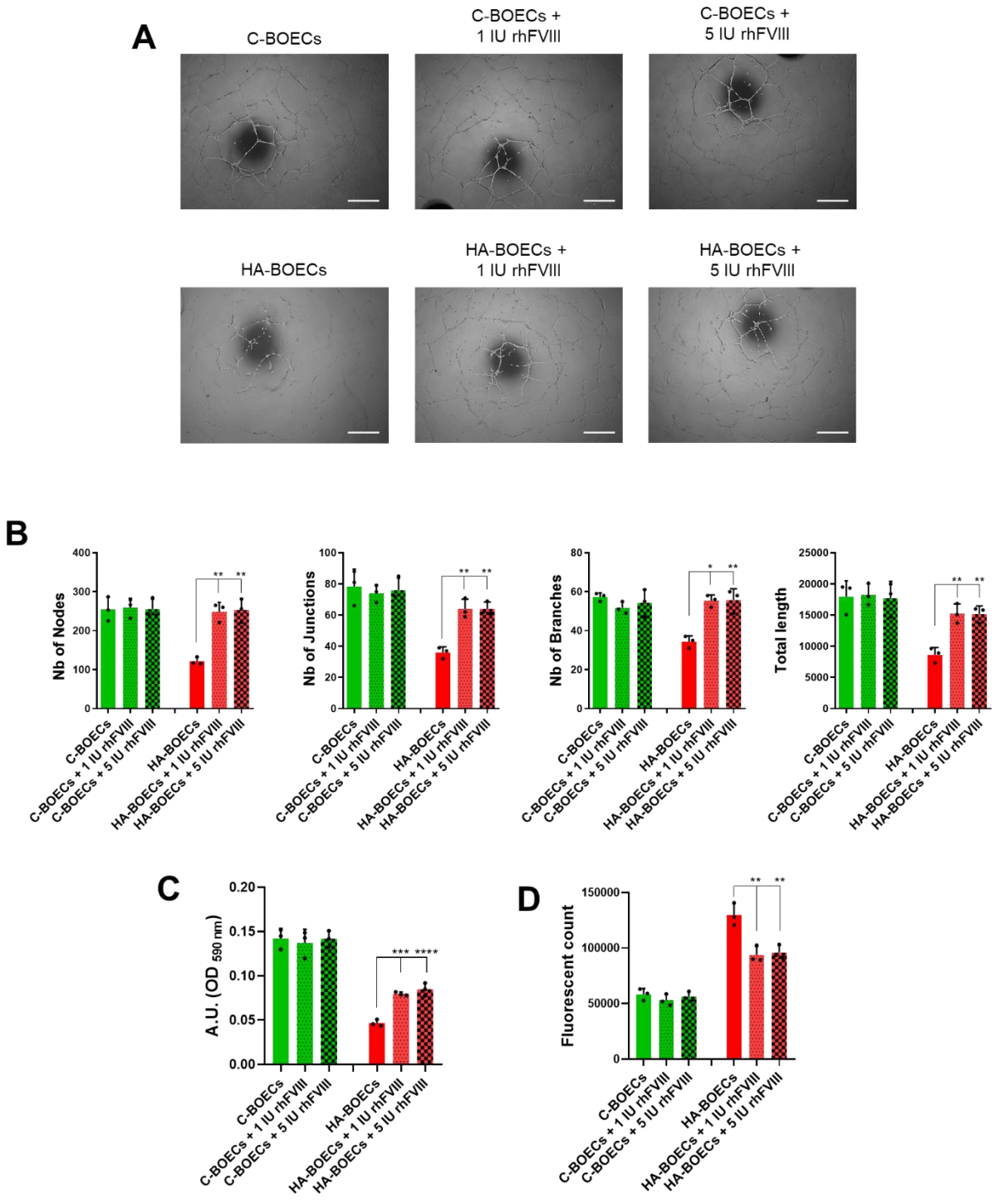


Figure 12. FVIII treatment of HA-BOECs enhances EC function
A Representative images from a tubulogenic assay on C (n=3) and HA-BOECs (n=3) treated with 1 or 5 IU/ml of rhFVIII. Scale bar = 500 µm. **B** Quantitative analysis of the number of nodes, junctions, branches, and total length of tubule networks. **C** Indirect measurement of cell migration through crystal violet staining elution. **D** Quantification of permeability based on the extravasation of FITC-dextran through an intact monolayer. Data in **B**, **C**, and **D** are expressed as mean ± SD. All experiments were performed once for each subject (3 for each group). Statistical analysis was performed by one-way ANOVA test (***p < 0.001; **p < 0.01; *p < 0.05).

FVIII is required for *in vivo* vessel integrity maintenance

We next analyzed *in vivo* vessel permeability by injecting NSG and NSG-HA mice intravenously with Evans blue, an albumin-binding dye. Typically, under physiological conditions the endothelium is impermeable to albumin, keeping Evans blue confined within the blood vessels (Figure 13, first panel). However, increased endothelial permeability in NSG-HA mice allowed for the extravasation of Evans blue into the surrounding tissues (Figure 13, second panel). In line with our previous findings, LV-FVIII NSG-HA mice, assessed 50 days after receiving $5x10^8$ TU/mouse of LV-FVIII, showed a marked reduction in dye extravasation (Figure 13, third panel). Similarly, NSG-HA mice treated every two days with 4 IU of rhFVIII for 20 days showed restored physiological vascular permeability, as judged by a significant decrease in Evans blue leakage (Figure 13, fourth panel). Taken together, these results indicate that FVIII plays a crucial role in *in vivo* vessel integrity.

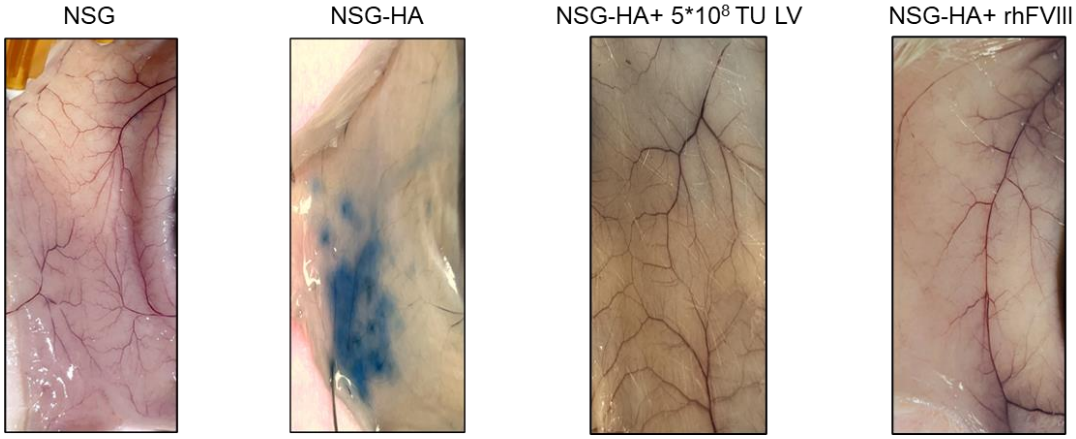


Figure 13. FVIII regulates vascular *in vivo* permeability
Representative images show Evans blue dye extravasation in interstitial tissues of NSG (first panel) and NSG-HA mice (second panel), NSG-HA mice injected with LV- FVIII (third panel), and NSG-HA treated with rhFVIII (fourth panel). The total number of animals used for each condition was 6 except for NSG-HA mice injected with LV- FVIII, where 4 mice were used.

Knockout-*F8* ECs show a significant reduction in functionality

To confirm FVIII involvement in EC functionality and stability, we used CRISPR/Cas9 technology to target *F8* in ECs. Firstly, we transduced human microvascular endothelial cells (HMEC-1) with a two-vector CRISPR/Cas9 system with a doxycycline inducible Cas9 (LV-

Cas9), and a second construct carrying the guide RNA (gRNA) of interest (Figure 9). According to previous results, two guides were chosen for these experiments: one targeting exon 4 (gRNA 1) and the other targeting exon 7 (gRNA 2).

As shown in Figure 14A both gRNA LVs transduced ECs with high efficiency. After the treatment with doxycycline, we observed an increase in clotting time (Figure 14B) that correlated with decreased FVIII levels compared to control cells (Figure 14C). Even if both gRNAs were able to significantly reduce FVIII protein levels, gRNA 1 was the most efficient. We performed endothelial functional assays on these cells which showed a decreased tubule formation, migration capability and an altered permeability compared to control (Figure 14D-G). These data furtherly confirmed that ECs are regulated by FVIII and its absence determines a drastic functional impairment. Remarkably, all these functions were significantly rescued after the treatment with rhFVIII (Fig 14D-G) highlighting the potential role of prophylaxis in restoring HA EC impairment.

More importantly, we confirmed these data also on C-BOECs which were transduced with the same CRISPR/Cas9 system (Fig 15A). The higher efficiency of gRNA 1 was more evident in these cells resulting in a greater reduction of secreted FVIII by BOECs transduced with this guide (KO-*F8* BOECs 1) compared to those transduced with gRNA 2 (KO-*F8* BOECs 2) (Fig 15B, C). Interestingly, we found that a lower FVIII expression correlates with an enhanced impairment of EC functions. As for HMEC-1, KO-*F8* C-BOECs show a significant impairment in their tubulogenic potential and migratory capacity as well as they display an altered permeability. Finally, rhFVIII treatment improved their functionality in all the performed assays (Fig 15D-G).

In conclusion, these data reveal a new function for FVIII, suggesting that beyond its established role in coagulation, it directly contributes to enhancing EC functionality.

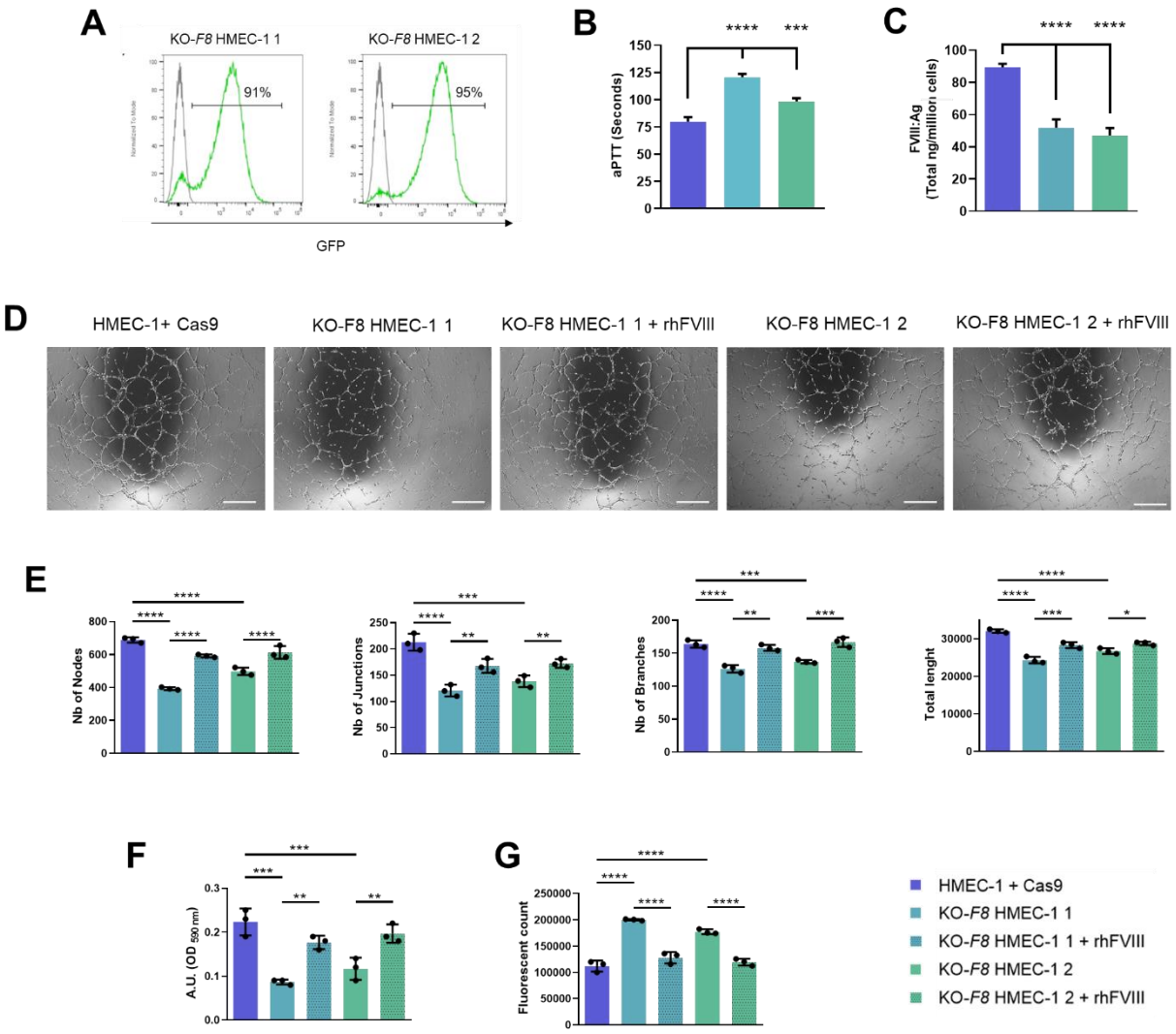


Figure 14. FVIII knockout on HMEC-1 induces functional impairments

A Representative histograms for GFP evaluation by FACS analysis in KO-*F8* HMEC-1. HMEC-1+Cas9 were used as control. **B** aPTT assay on supernatant of KO-*F8* HMEC-1. HMEC-1+Cas9 were used as control. **C** FVIII antigen assay on supernatant of KO-*F8* HMEC-1. **D** Representative images from a tubulogenic assay on KO-*F8* HMEC-1 in presence or absence of 1 UI/mL rhFVIII**.** Scale bar = 500 µm. **E** Quantification of number of nodes, junctions, branches and total length of the tubule networks of samples in E. **F** Indirect measurement of cell migration through crystal violet staining elution of samples in E. **G** Quantification of permeability based on the extravasation of FITC-dextran through an intact monolayer of samples in E. Data in **B**, **C**, **D**, **E**, and **G** are expressed as mean ± SD. All experiments were performed three times. Statistical analysis was performed by one-way ANOVA test (**** $p<0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

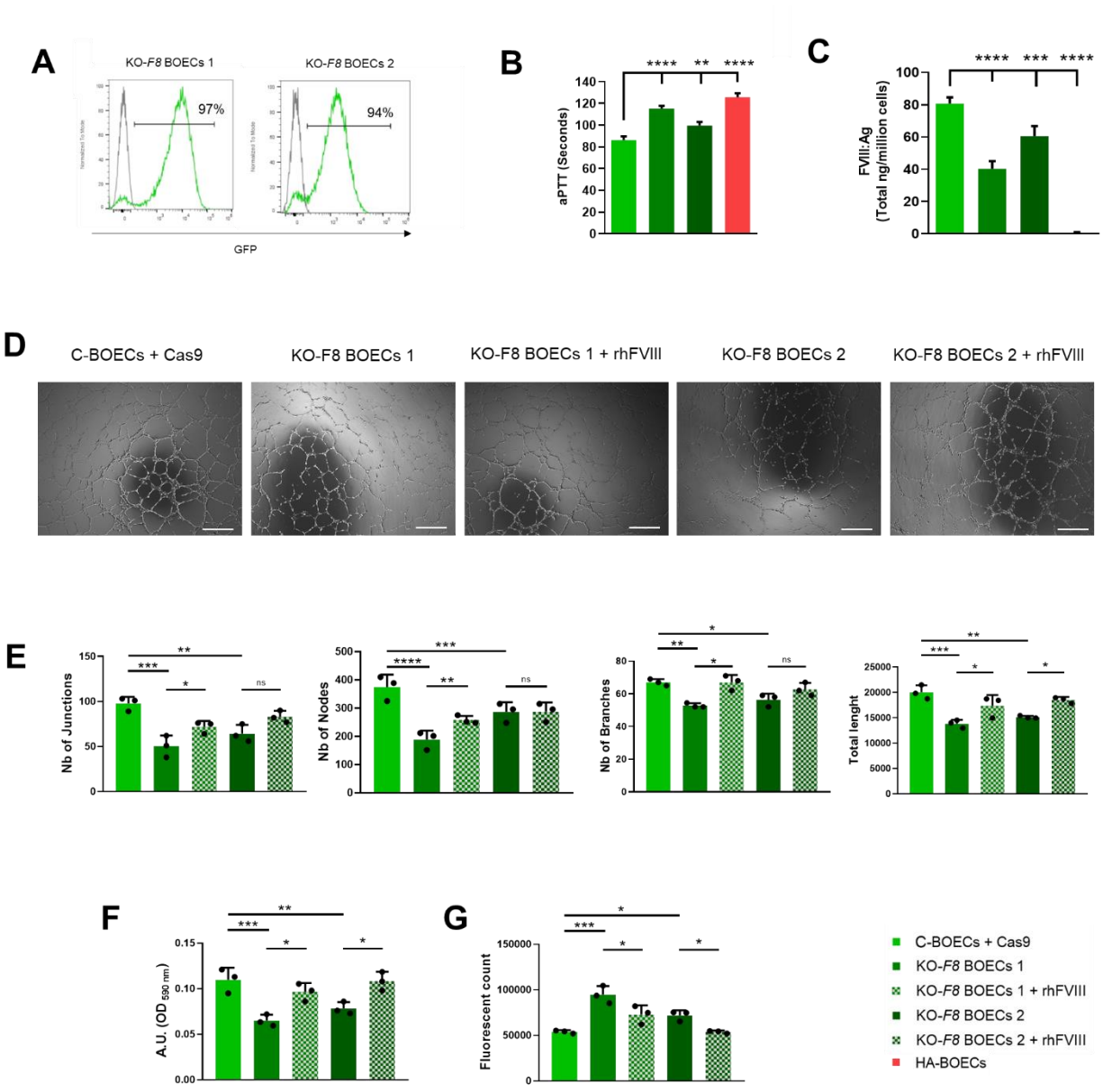


Figure 15. KO-F8 BOECs are defective in tubulogenesis and migration and show an altered permeability
A Representative histograms for GFP evaluation by FACS analysis in KO-*F8* C-BOECs. **B** aPTT assay on supernatant of KO-*F8* C-BOECs. C-BOECs+Cas9 and HA-BOECs were used as controls. **C** FVIII antigen assay on supernatant of KO-*F8* C-BOECs. C-BOECs+Cas9 and HA-BOECs were used as controls. **D** Representative images from a tubulogenic assay on KO-*F8* C-BOECs in presence or absence of 1 UI/mL rhFVIII**.** Scale bar = 500 µm. **E** Quantification of number of nodes, junctions, branches and total length of the tubule networks of samples in D. **H** Indirect measurement of cell migration through crystal violet staining elution of samples in D. **D** Quantification of permeability based on the extravasation of FITC-dextran through an intact monolayer of samples in D. Data in **B**, **C**, **E**, **F**, and **G** are expressed as mean ± SD. All experiments were performed three times. Statistical analysis was performed by one-way ANOVA test (**** $p<0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$).

FVIII binding to integrin β1 activates the FAK pathway, regulating *in vitro* EC behavior

We next investigated the role of FVIII in regulating signaling pathways involved in angiogenesis, migration, and EC functionality. For this purpose, we compared the activation of tyrosine-phosphorylation pathway by *in vitro* rhFVIII administration with that induced by recombinant human vascular endothelial growth factor (rhVEGF), a well-known regulator of ECs. We found that treating HA-BOECs with either rhFVIII or rhVEGF for 15 min resulted in similar phosphorylation levels of a 120 kDa protein (Figure 16A). However, unlike rhVEGF, rhFVIII failed to induce the phosphorylation of VEGF receptor 2 (VEGFR-2) and ERK (Figure 16B), indicating that FVIII operates via a distinct pathway. Thus, we focused on the 120 kDa phosphorylated protein, which we identified as focal adhesion kinase (FAK); we also found that FVIII treatment induced the phosphorylation of Src along with their downstream targets AKT, mTOR, and p38, but not ERK (Figure 16C, D).

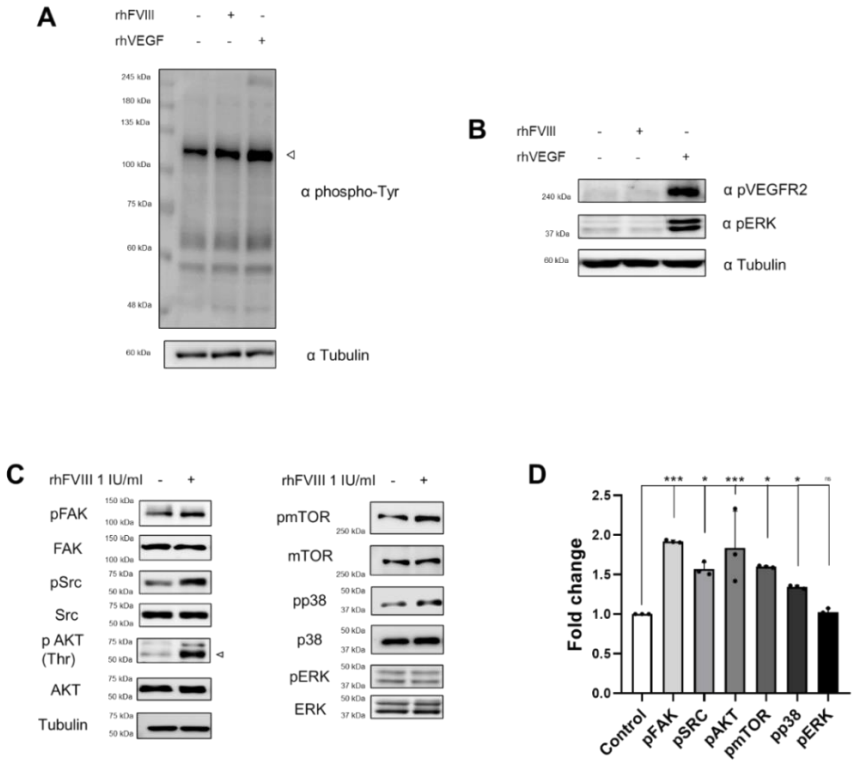


Figure 16. FVIII induces activation of FAK/Src pathway
A Western blot analysis of whole cell lysates (WCL) from HA-BOECs incubated for 15 min in the presence or absence of rhFVIII (1 IU/ml) or rhVEGF (50 ng/ml) and stained with antibody against phospho-tyrosines. Tubulin was used as a loading control. **B** Western blot analysis of WCL from HA-BOECs incubated for 15 min in the presence or absence of rhFVIII (1 IU/ml) or rhVEGF (50 ng/ml) and stained with antibody against pVEGFR2 and pERK. Tubulin was used as a loading control. **C** Western blot analysis of WCL from HA-BOECs treated for 15 min with or without 1 IU/ml of rhFVIII and stained with antibodies against pFAK, total FAK, pSrc, total Src, pAKT, total AKT, pmTOR, total mTOR, pp38, total p38, pERK, and total

ERK. Tubulin was used as a loading control. **D** Quantification of increased phosphorylation of FAK, Src, AKT, mTOR, and p38 expressed as fold change relative to untreated control. Data are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA test (***p < 0.001; *p < 0.05)

To investigate whether this signaling was a direct consequence of FVIII binding with a surface receptor on ECs, we used the Flow-TriCEPS ligand-based receptor capture technology. This assay is based on the use of a three-arm ligand-receptor-capturing system that allows the detection of ligand-receptor binding on living cell membranes via FACS analysis. HA-BOECs were treated with 10 IU/ml rhFVIII for 1 h in the presence of the Flow-TriCEPS system. As shown in Figure 17A, the rhFVIII-TriCEPS complex was able to bind to HA-BOECs with higher intensity compared to the negative control, glycine-TriCEPS, and even to the positive control, transferrin-TriCEPS, implying that FVIII interacts with a/multiple receptor/s on the surface of these cells.

Based on our results, we turned our attention to integrin β1 (ITGB1), a key EC molecule involved in angiogenesis, known for its ability to induce FAK phosphorylation. Interestingly, we observed that ITGB1 indeed co-immunoprecipitated with FVIII in a dose-dependent manner (Figure 17B). To further confirm this FVIII-ITGB1 interaction, we performed co-immunoprecipitation experiments in HA-BOECs where ITGB1 was silenced using two different short hairpin RNAs (shRNAs) (Figure 17D). The FVIII signal was significantly reduced in these silenced-ITGB1 cells, suggesting that in absence of its receptor, FVIII precipitates less, confirming FVIII-ITGB1 interaction (Figure 17C). Therefore, we found that FVIII was unable to increase FAK phosphorylation in HA-BOECs, either silenced for ITGB1 or treated with Defactinib, a specific FAK inhibitor (FAKi), (Figure 17E, F), reinforcing the role of FVIII in regulating the ITGB1-FAK axis.

To determine the need of FVIII-mediated activation of ITGB1-FAK signaling for EC functionality, we performed tubulogenesis, migration, and permeability assays on HA-BOECs that were either silenced for ITGB1 or treated with FAKi, and then exposed to rhFVIII. We observed that FAKi treatment significantly disrupted the FVIII-dependent angiogenic response. Congruently, ITGB1-silenced HA-BOECs failed to form tubules *in vitro*, showing no improvement even upon FVIII treatment (Figure 18A, B). Similar results were obtained in migration and permeability assays (Figure 18C, D). Overall, these data demonstrate that FVIII directly interacts with ITGB1, thereby triggering downstream signaling crucial for maintaining EC functionality.

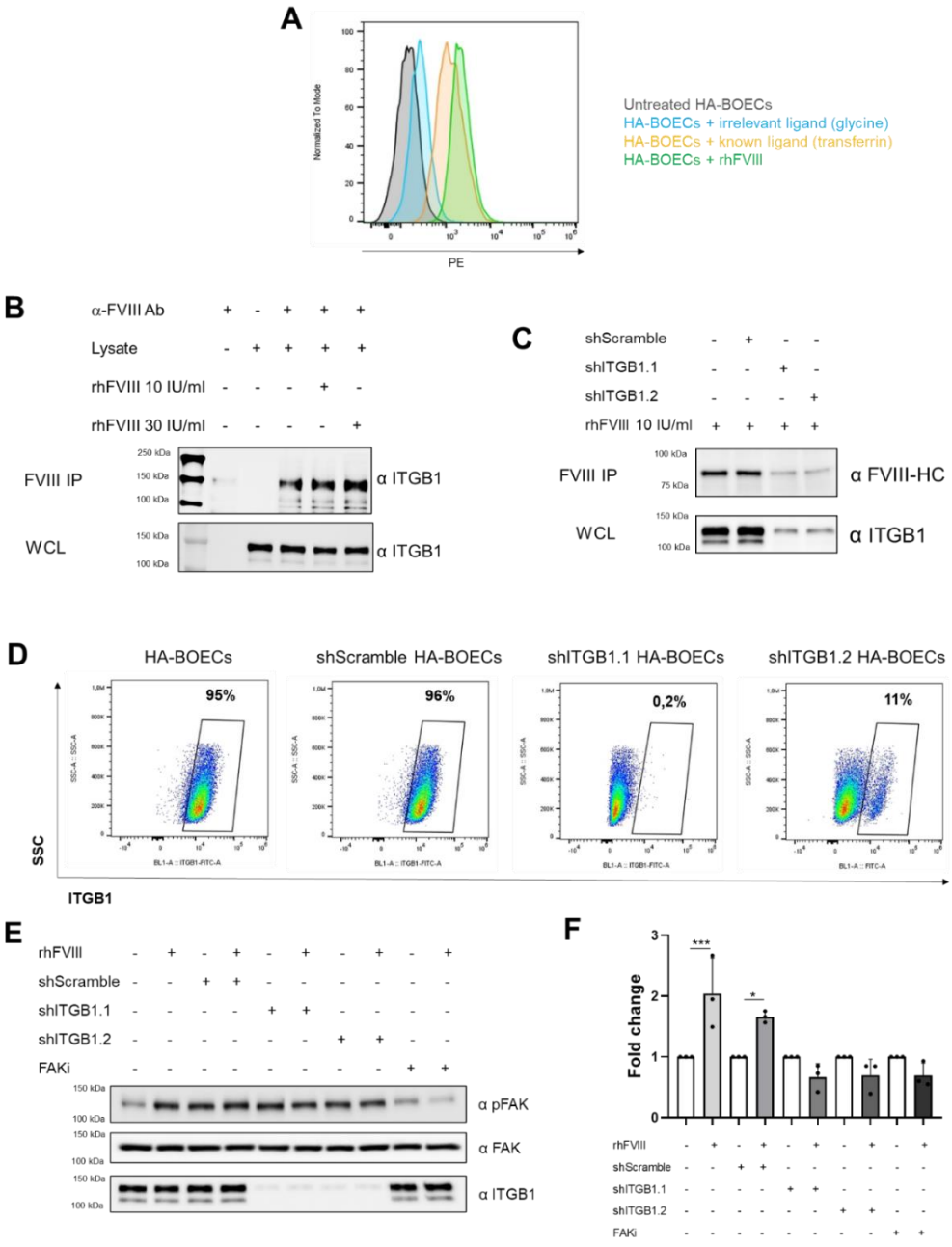


Figure 17. FVIII binds integrin β1 inducing FAK phosphorylation
A FACS analysis of HA-BOECs treated with glycine-TRiCEPS (irrelevant ligand, negative control), transferrin-TRiCEPS (known ligand, positive control), or rhFVIII-TRiCEPS (rhFVIII). Cells stained only with streptavidin-PE were used as an additional control (untreated HA-BOECs). **B** Western blot analysis of ITGB1 on an immunoprecipitation for FVIII of HA-BOECs treated for 1 h with either 10 IU/ml or 30 IU/ml of rhFVIII, alongside WCL. **C** Western blot showing the heavy chain of FVIII in HA-BOECs treated for 1 h with 10 IU/ml of rhFVIII following immunoprecipitation for FVIII (FVIII IP). WCL were also probed with an antibody anti-ITGB1. **D** Dot plots from flow cytometry analysis showing ITGB1 expression levels in HA-BOECs, shScramble, shITGB1.1 and shITGB1.2 HA-BOECs. **E** Western blot analysis of WCL from FAKi-treated HA-BOECs, as well as HA-BOECs transduced with lentiviral vectors (LV) carrying either shScramble, shITGB1.1, or shITGB1.2. All cells were incubated for 15 min in the presence or absence of 1 IU/ml of rhFVIII and stained with antibodies against pFAK, total FAK, or ITGB1. **F** Quantification of the increase in FAK phosphorylation, expressed as fold change relative to each untreated control. Data are expressed as mean ± SD and one-way ANOVA test was performed (***$p < 0.001$; *$p < 0.05$).

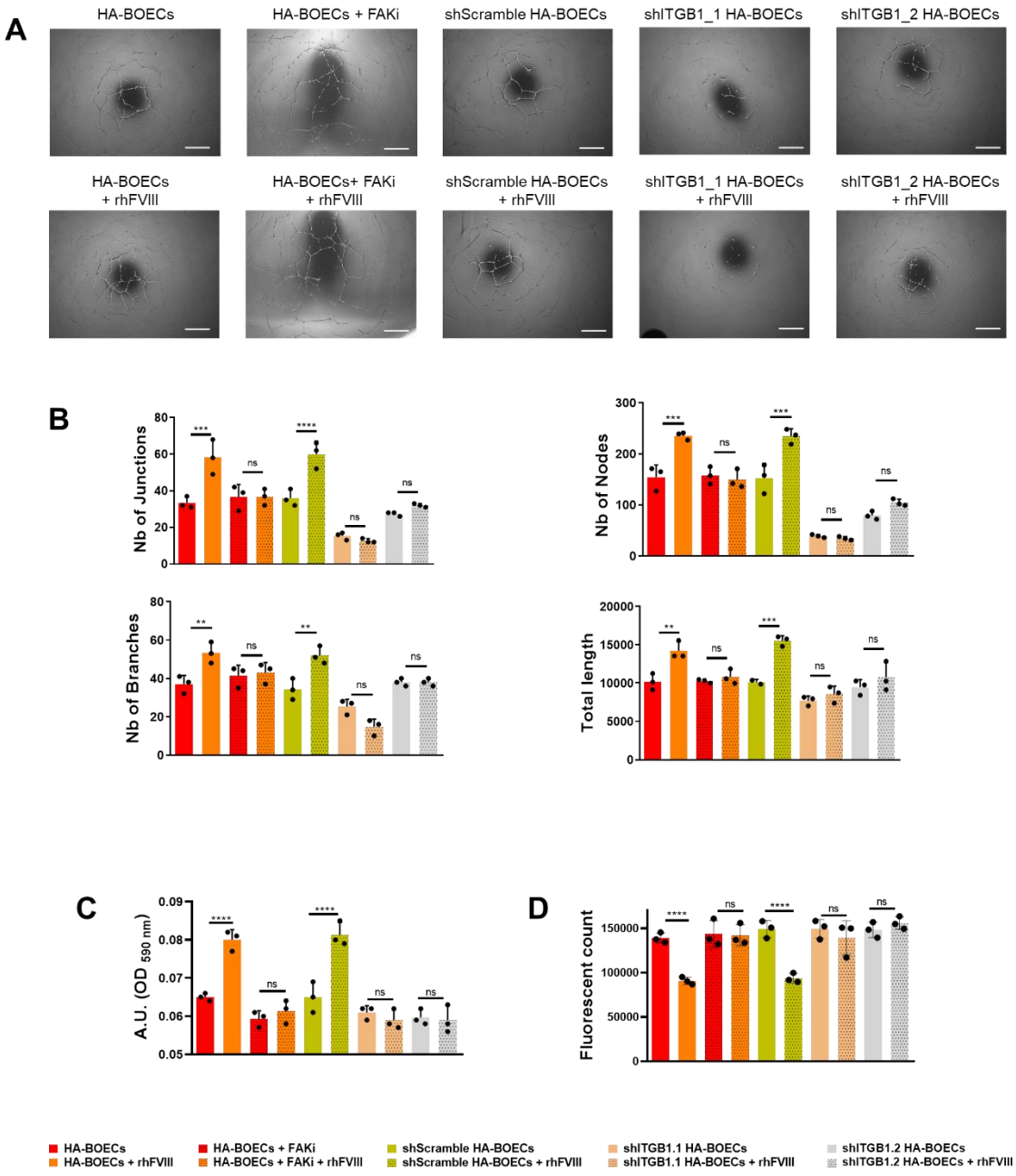


Figure 18. FVIII-Integrin β1 binding activates FAK pathway regulating EC functions
A Representative images from a tubulogenic assay performed on untreated or FAKi-treated HA-BOECs, as well as HA-BOECs transduced with lentiviral vectors (LV) carrying either shScramble, shITGB1.1, or shITGB1.2. All cells were incubated in the presence or absence of 1 IU/mL rhFVIII. Scale bar = 500 µm. **B** Quantitative analysis of the number of nodes, junctions, branches, and total length of tubule networks in HA-BOECs treated as described in A. **C** Indirect measurement of cell migration by elution of crystal violet staining in HA-BOECs treated as described in A. **D** *In vitro* permeability assay quantification calculated on the extravasation of FITC-dextran through an intact monolayer of HA-BOECs treated as described in a. Data in **B**, **C**, and **D** are expressed as mean ± SD. All the experiments were performed three times. Statistical analysis was performed by one-way ANOVA test (****p < 0.0001; ***p < 0.001 **p < 0.01).

FVIII regulates genes involved in extracellular matrix organization

To explore the influence of FVIII on the transcriptomic profile of ECs, we performed RNA sequencing (RNASeq) experiments, comparing HA to control BOECs in an unpaired analysis, and comparing LV-FVIII HA to HA-BOECs in a paired analysis. Analysis of the data using volcano plots revealed 155 genes that were downregulated in HA compared to C-BOECs, with a LogFC > 2 and p-value < 0.01. These genes primarily participate in extracellular matrix (ECM) composition, such as collagen4a1 (COL4A1), nidogen2 (NID2), fibulin1 (FBN1), and peroxidasin (PXDN) (Figure 19A). Notably, the reintroduction of FVIII in HA-BOECs led to a significant restoration in the expression levels of most of these genes (Figure 19B, C).

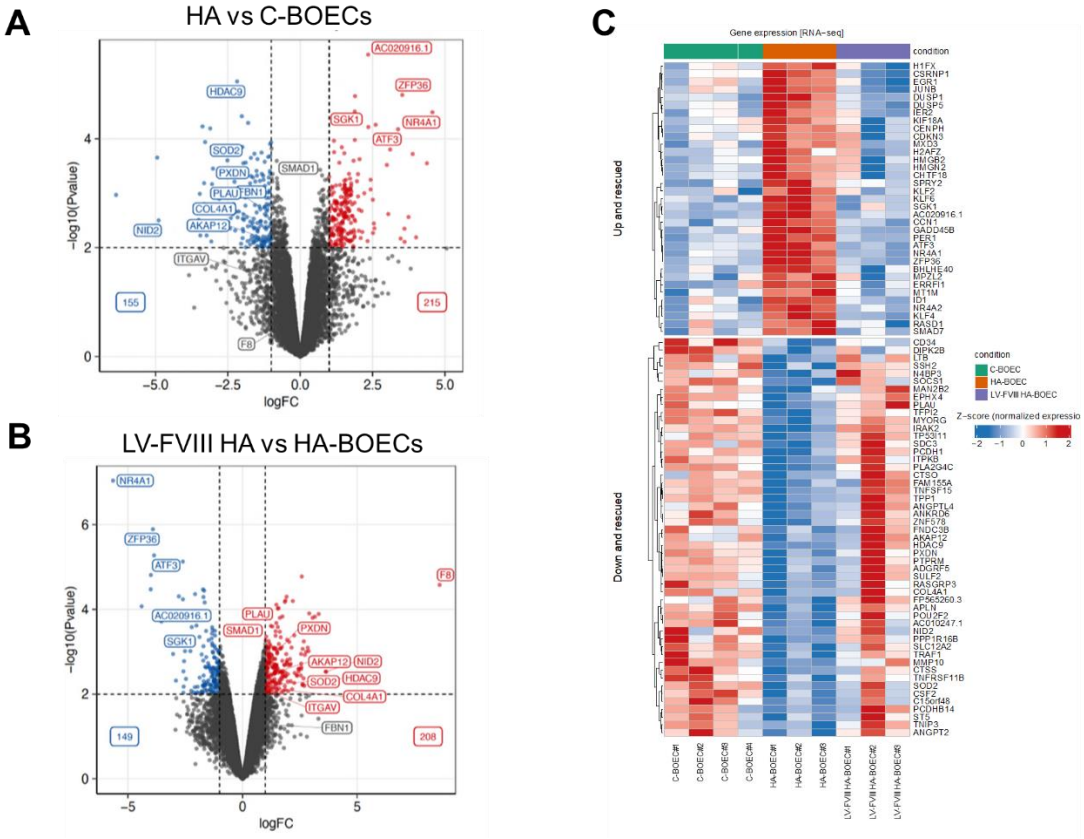


Figure 19. FVIII regulates the expression of genes related to EC functionality
A Volcano plot showing differentially expressed genes (DEGs) in HA *vs* C-BOECs. Genes are classified as up-regulated if they have a log fold change > 1 and p-value < 0.01, and as down-regulated if they have a log fold change < -1 and p-value < 0.01. **B** A similar volcano plot for LV-FVIII HA-BOECs vs HA-BOECs. **C** Heatmap showing the expression pattern of genes rescued by LV transduction. These are genes significantly differentially expressed both in HA vs C-BOECs and in LV-FVIII HA-BOECs vs HA-BOECs, but exhibit a reversal in the sign of log fold change: down-regulated in the disease condition and up-regulated following transduction, or *vice versa*.

In agreement with our *in vitro* and *in vivo* findings, gene set enrichment analysis (GSEA) of differentially expressed genes (DEGs), identified pathways corresponding to vascular development, cell migration, regulation of cell adhesion, extracellular matrix organization, and integrin cell surface interactions (Figure 20A). These pathways were downregulated in HA vs C-BOECs and were rescued upon LV-FVIII transduction. Similar results were obtained with gene ontology (GO) process analysis of DEGs, revealing a common cluster of the modulated genes in a protein-protein interaction (PPI) network. The most enriched gene sets in the GO analysis were very similar to those identified by GSEA, including fundamental EC functions, such as angiogenesis, cell-adhesion, and ECM organization, further corroborating our findings (Figure 20B).

Together, these results uncover a unique transcriptomic profile regulated by FVIII expression in BOECs. This profile aligns with the *in vitro* and *in vivo* functional differences described above among C, HA, and LV-FVIII HA-BOECs.

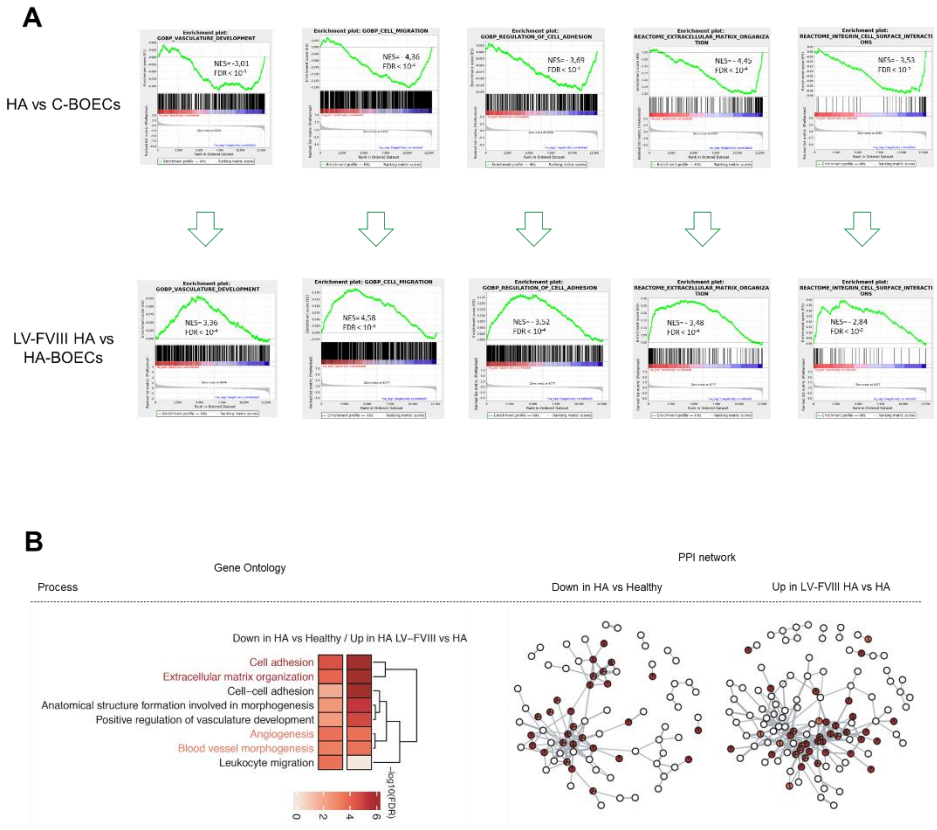


Figure 20. GSEA and Gene Ontology analysis of FVIII-modulated genes
A Gene set enrichment analysis (GSEA) plots depicting pathways that are downregulated in HA vs C-BOECs and rescued in LV-FVIII HA vs HA-BOECs. Key pathways include vascular development, cell migration, regulation of cell adhesion, extracellular matrix (ECM) organization and integrin cell surface interactions. **B** On the left panel, a heatmap shows the top

commonly enriched gene ontology (GO) process terms among the differentially expressed genes (DEGs) identified by RNAseq. These DEGs are compared across two categories: "Down in HA vs Healthy" and "Up in HA LV-FVIII vs HA". Hierarchical clustering was applied using the log10-adjusted p-values from the enrichment analysis. On the right panel, the STRING protein-protein interaction (PPI) network illustrates the interactions among these DEGs. Nodes within the network are color-coded to correspond with the commonly enriched GO annotations highlighted on the right.

FVIII enhances the expression of NID2 preserving EC functions

Our transcriptomic analysis revealed that FVIII plays a key role in regulating genes essential for ECM organization in ECs (Figure 20). To gain more mechanistic insights, we focused on NID2, a glycoprotein crucial for endothelial basement membrane stability, which was one of the most significantly downregulated gene in HA vs C-BOECs and rescued by LV-mediated transduction of FVIII (Figure 21A, B). Importantly, time-course analysis showed that NID2 expression was induced upon FVIII treatment after 48h and FAKi prevented the FVIII-induced increase in NID2 expression (Figure 21C).

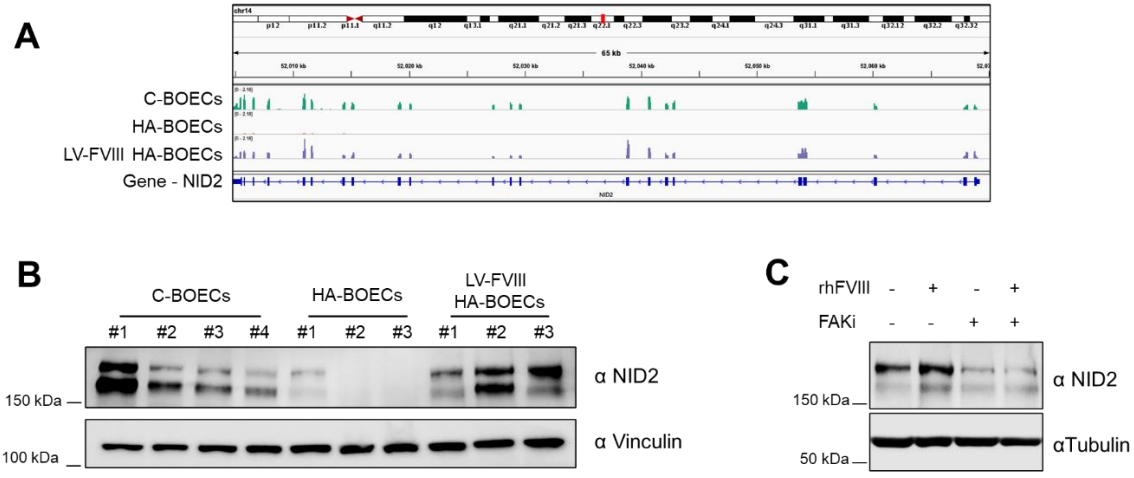


Figure 21. NID2 is a downstream effector of FVIII signalling through FAK
A Genome browser view showing the average RNAseq signal profile of the NID2 gene in C, HA, and LV-FVIII HA-BOECs. **B** Western blot analysis of NID2 expression levels in C (n=4), HA (n=3), and LV-FVIII HA-BOECs (n=3). An anti-vinculin antibody was used to confirm equal loading. **C** Western blot analysis of NID2 expression in HA-BOECs treated or not with rhFVIII in the presence or absence of defactinib (FAKi) for 48 h. An anti-tubulin antibody was used as loading control.

Thus, we transduced HA-BOECs with LV carrying NID2 to enhance its expression in those cells that physiologically show a low expression of NID2 (Figure 22A). Interestingly, we found that ectopic expression of NID2 in HA-BOECs not only enhanced their tubulogenic functionality (Figure 22B, C) but also increased their migratory capacity (Figure 22D) and restored barrier integrity to levels comparable to those seen in C-BOECs (Figure 22E).

To substantiate the role of NID2 in EC functionality, we silenced NID2 expression using a specific shRNA both in HMEC-1 (Figure 23A) and C-BOECs (Figure 24A). NID2 knockdown significantly impaired EC tubulogenesis (Figure 23B, C and 24B, C), and migration (Figure 23D and 24D), while it increased EC permeability (Figure 23E and 24E). Importantly, treatment with rhFVIII failed to restore the impaired functions in these NID2-deficient ECs (Figure 23B-E and 24B-E), thus confirming that NID2 is required for FVIII-mediated regulation of fundamental EC processes.

Overall, these findings establish a crucial link between FVIII and EC functionality, underscoring how ECs respond to FVIII by downstream signaling pathways crucial in regulating their behavior.

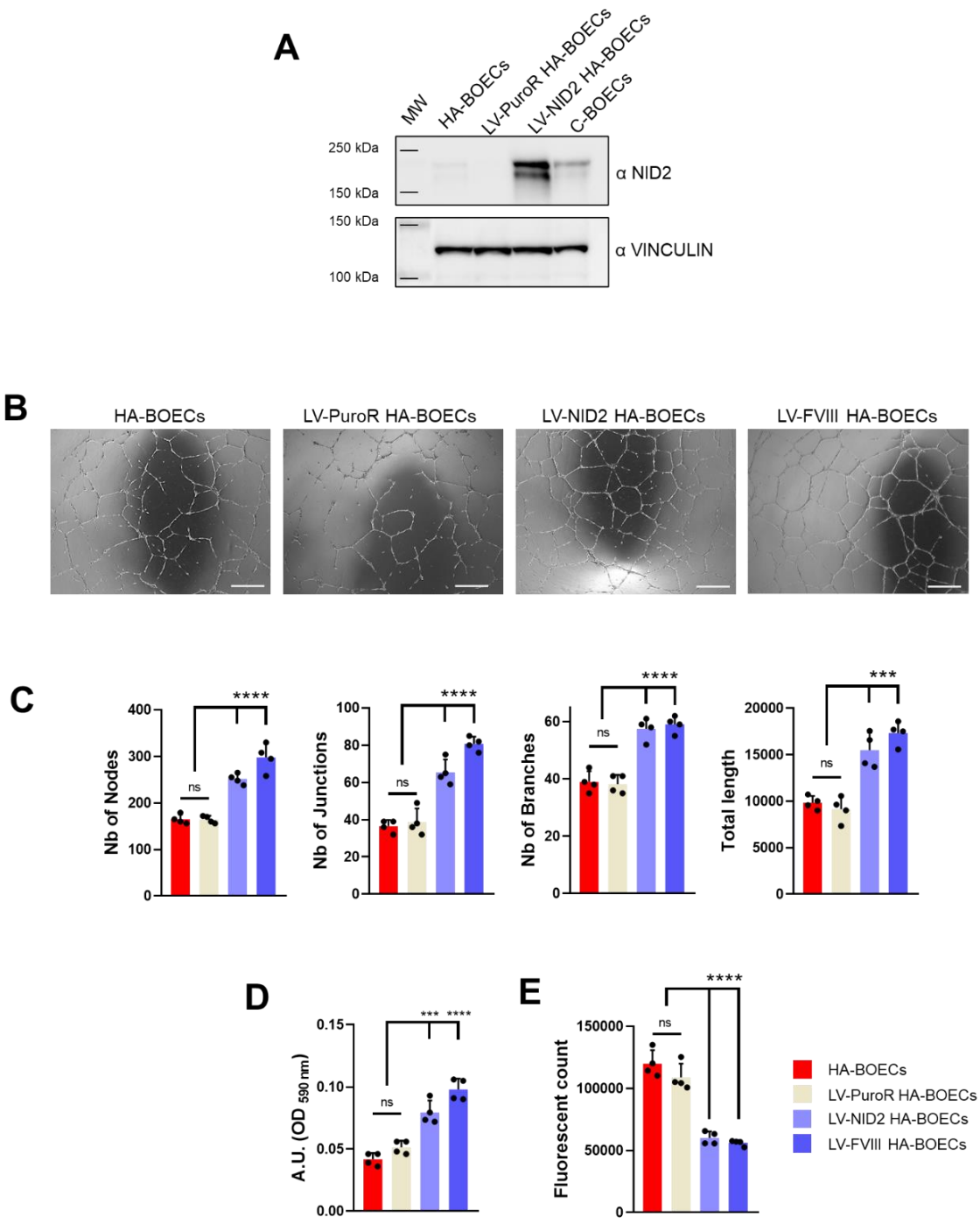


Figure 22. Overexpression of NID2 in HA-BOECs improves *in vitro* functionality
A Western blot comparison of NID2 levels in LV-NID2 HA-BOECs vs HA-BOECs, with LV.PuroR HA-BOECs and C-BOECs used as controls. **B** Representative images from a tubulogenic assay conducted on samples described in A. Scale bar = 500 µm. **C** Quantification of number of nodes, junctions, branches, and total tubule network length of tubule networks as described in A. **D** Indirect measurement of cell migration through crystal violet staining elution in samples as described in A. **E** Permeability assay results, based on the extravasation of FITC-dextran through an intact monolayer in the BOEC groups, as described in A. Data in **C**, **D**, and **E** are expressed as mean ± SD. All the experiments were performed four times. Statistical analysis was performed by one-way ANOVA test (****$p < 0.0001$; ***$p < 0.001$).

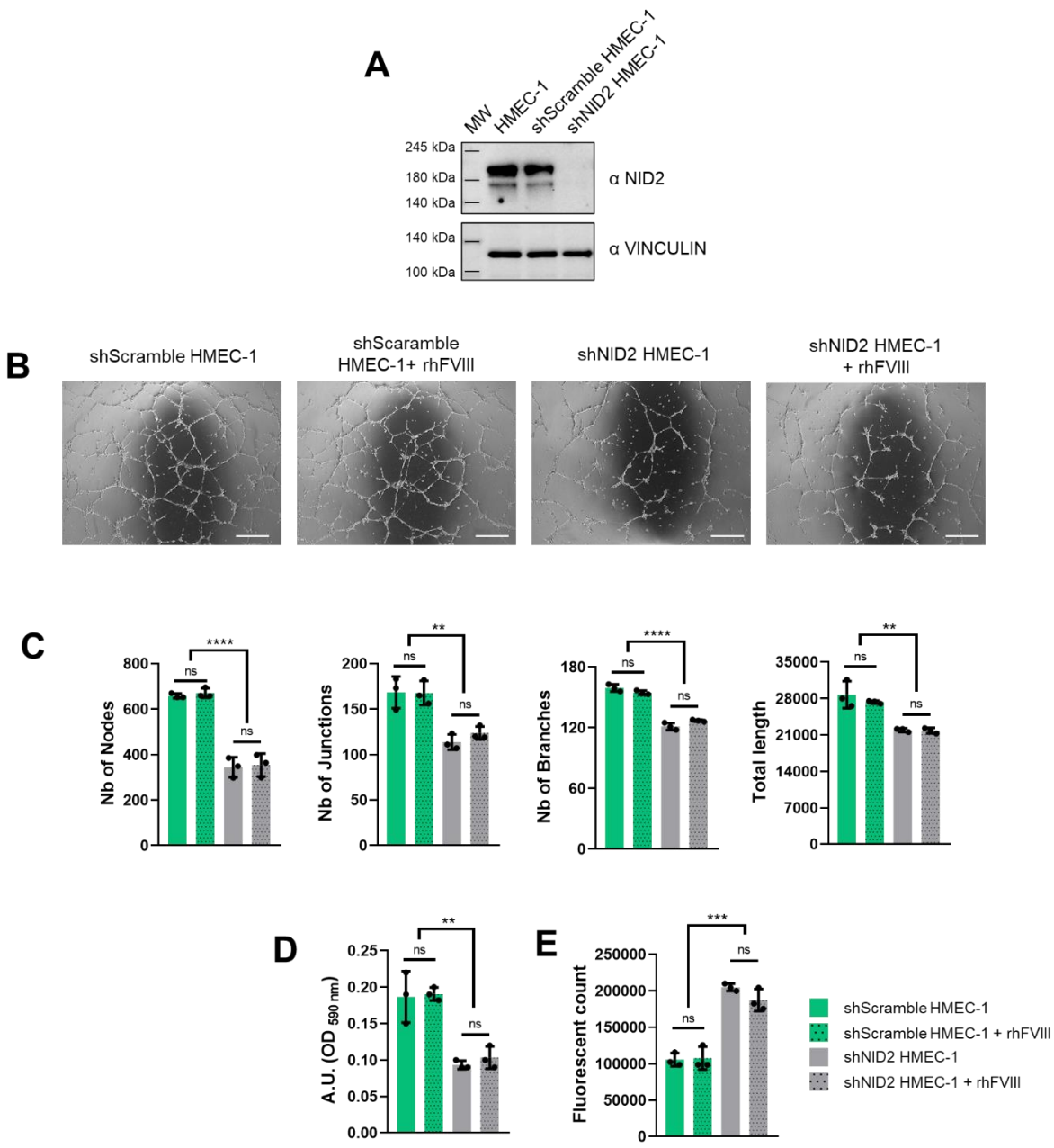


Figure 23. NID2 knockdown impairs HMEC-1 endothelial functionality
A Western blot analysis depicting NID2 expression in shNID2 HMEC-1 cells compared to NT or shScramble-transduced HMEC-1. Vinculin was used as loading control. **B** Representative images from a tubulogenic assay on shScramble and shNID2 HMEC-1, both with and without rhFVIII treatment (1 IU/ml). Scale bar= 500 µm. **C** Quantitative analysis of the number of nodes, junctions, branches, and total length of the tubule networks of the samples as described in B. **D** Indirect measurement of cell migration through the elution of crystal violet staining of the samples as described in B. **E** Permeability assay quantification, based on the extravasation of FITC-dextran through an intact monolayer of the samples as described in B. Data in **C**, **D**, and **E** are expressed as mean ± SD. All the experiments were performed three times. Statistical analysis was performed by one-way ANOVA test (****$p < 0.0001$; **$p < 0.01$).

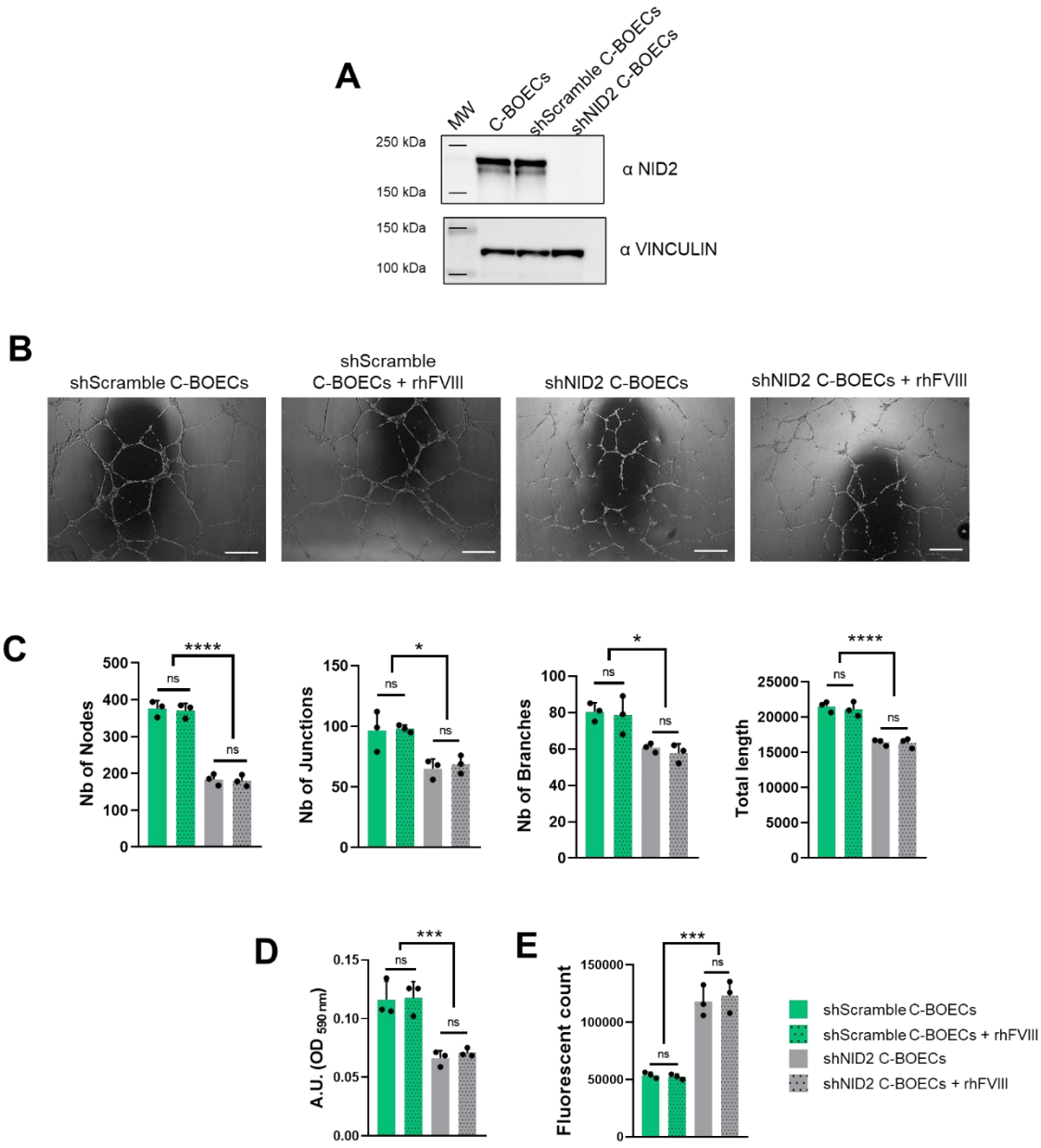


Figure 24. Knockdown of NID2 in C-BOECs impairs their functionality
A Western blot analysis depicting NID2 expression in shNID2 C-BOECs cells compared to NT or shScramble-transduced C-BOECs. Vinculin was used as loading control. **B** Representative images from a tubulogenic assay on shScramble and shNID2 C-BOECs, both with and without rhFVIII treatment (1 IU/ml). Scale bar= 500µm. **C** Quantitative analysis of the number of nodes, junctions, branches, and total length of the tubule networks of the samples as described in B. **D** Indirect measurement of cell migration through the elution of crystal violet staining of the samples as described in B. **E** Permeability assay quantification, based on the extravasation of FITC-dextran through an intact monolayer of the samples as described in B. Data in **C**, **D**, and **E** are expressed as mean ± SD. All the experiments were performed three times. Statistical analysis was performed by one-way ANOVA test (****$p < 0.0001$; ***$p < 0.001$; * $p<0.05$).

Endothelial proteomic profile is highly modulated by FVIII

To investigate in depth the proteins that are modulated by the presence (or absence of FVIII), we performed a proteomic analysis on C, HA and LV-FVIII HA-BOECs (n=3 for each sample).

We found a profound difference in all the proteomic profiles that we analyzed. Specifically, we found 724 upregulated and 432 downregulated proteins in HA compared to C-BOECs (FC > 1.3 and p-value < 0.05), significantly suggesting that FVIII expression is essential for a physiological endothelial proteomic profile (Figure 25A, B). Similarly, the comparison between LV-FVIII HA and HA-BOECs revealed 504 upmodulated and 768 downmodulated proteins when FVIII is re-introduced (Figure 25C, D).

As depicted by the principal component analysis (PCA), HA a C-BOECs cluster separately while LV-FVIII HA-BOEC show an intermediate localization between the two other groups (Figure 26A). Indeed, the first principal component (PC) describes almost the 60% of difference between all the samples and LV-FVIII HA-BOECs mainly moves on that PC (x axis) towards C-BOECs. Interestingly, as performed for the transcriptomic analysis, we focused on the proteins which were downregulated in HA-BOECs and rescued in LV-FVIII HA-BOECs. We found 251 proteins that, according to our analysis, are upregulated by the autologous expression of FVIII (Figure 26B).

This set of proteins was analyzed with STRING and similar data of the transcriptomic analysis were obtained (Figure 26C). Indeed, the largest identified cluster is composed by proteins related to angiogenesis (GO:0001525), ECM organization (GO:0030198), and positive regulation of cell migration (GO:0030335) (red cluster). These data highly reinforce our previous functional experiments confirming an enhanced expression of proteins involved in the tubulogenic process and ECM stability when FVIII is expressed. Among these proteins, we found some of those that transcriptomic analysis already revealed, such as COL4A1 and PXDN, and others which strongly correlate with our functional observations, including fibronectin (FN1), collagen4a2 (COL4A2), procollagen galactosyltransferase 1 (COLGALT1), and, especially, perlecan (HSPG2) which is one of the principal structural components of the endothelial basement membrane with COL4A1 and NID2. Unfortunately, NID2 was not detected probably due to a limitation of this analysis which is not able to entirely describe the proteomic profile of our samples. Cluster analysis identified other three large set of proteins: two related to the glycosaminoglycan (GO:0006027) and carbohydrate metabolism

(GO:0097367) (yellow and pink clusters) and another enriched with proteins of the electron transport chain (GO:0022904) and cellular respiration (GO:0045333) (brown cluster) (Figure 26C). This last cluster shows a high *strength* and low *false discovery rate* which describe respectively how large and how significant the enrichment effect is, introducing the hypothesis that HA BOECs lacking FVIII could have an impairment at the level of mitochondria. Importantly, among the proteins that composed this cluster, we found superoxide dismutase 2 (SOD2), which is widely recognized as a pivotal regulator of the mitochondrial function. Overall, these proteomic data confirmed our previous observations paving the way for new discoveries in the intricate relation between FVIII and ECs.

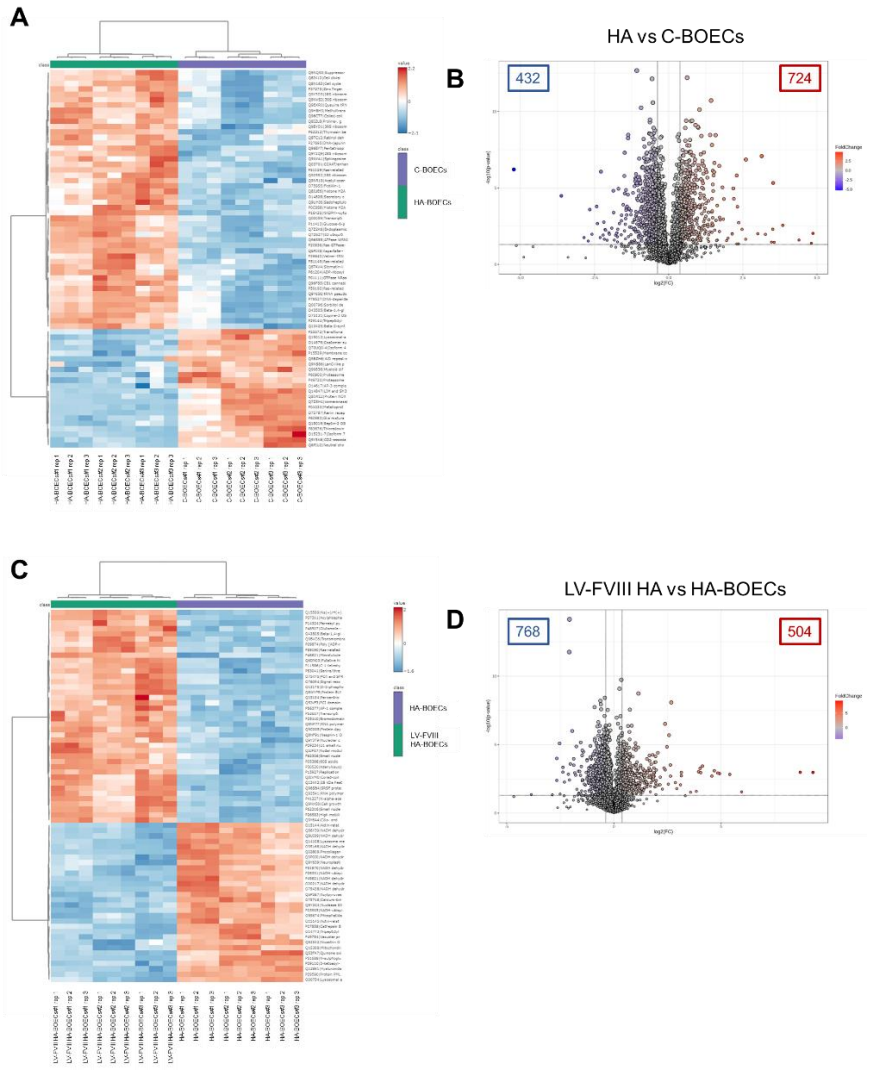


Figure 25. FVIII modulates proteomic profiles of BOECs
A Heatmap showing the expression pattern of differentially expressed proteins between HA vs C-BOECs. B Volcano plot showing the distribution of the same protein as A. **C** Heatmap showing the expression pattern of differentially expressed proteins between LV-FVIII HA vs HA-BOECs. **D** Volcano plot showing the distribution of the same protein as C.

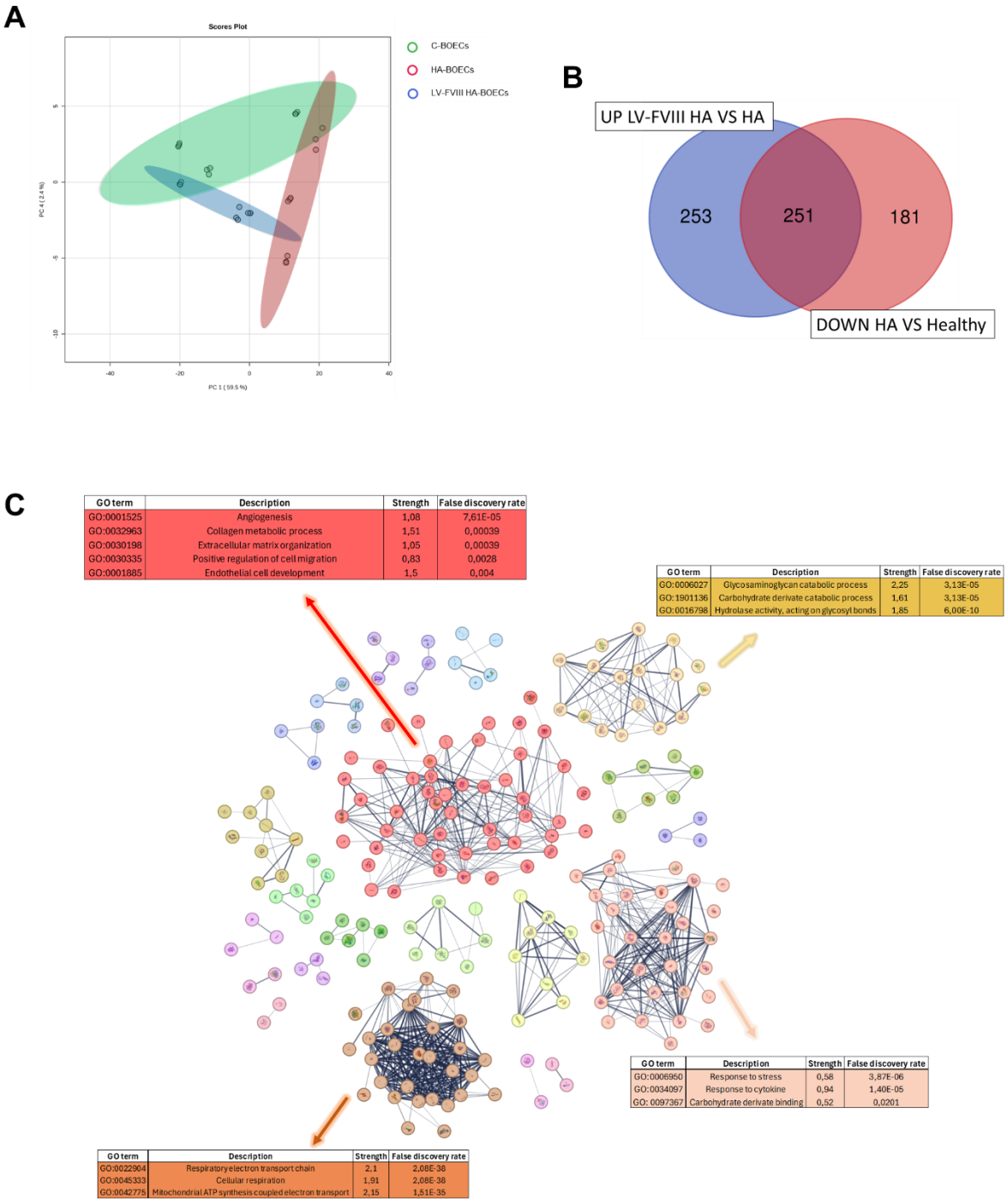


Figure 26. FVIII expression rescues proteins related to angiogenesis and cellular respiration
A Principal component analysis (PCA) describing the variation between the samples used for the proteomic analysis (C-BOECs in green, HA-BOECs in red and LV-FVIII HA-BOECs in blue). **B** Ven diagram representing the intersection between downregulated proteins in HA vs C-BOECs comparison and upregulated proteins in LV-FVIII HA vs HA-BOECs comparison. **C** Cluster analysis performed with STRING of the 251 proteins obtained in B. Only nodes belonging to a cluster are illustrate and only the four largest cluster have been analyzed to identify enriched gene ontology (GO) terms. In the reported tables are described GO terms, description, strength and false discovery rate.

Mitochondrial respiration is regulated by FVIII

To evaluate whether the HA-BOECs have compromised mitochondrial function compared to C-BOECs, we measured oxidative respiration in intact, non-permeabilized cells. Thanks to the Oroboros analysis we observed that HA-BOECs displayed a decrease in oxygen consumption in the three distinct phases of the mitochondrial respiration analysis (Figure 27A): routine (R), leakage (L) and maximum respiratory capacity in (ETS), measured upon treatment with FCCP, a protonophore that uncouples oxidation from phosphorylation. LV-FVIII HA-BOECs show a significant rescue in the oxygen consumption in the R and E phases (Figure 27B). In line with these results, in HA-BOECs the ATP-linked respiration, obtained by subtracting the proton leak from the routine oxygen consumption, is strongly reduced and LV-FVIII partially recovers the oxygen consumption (Figure 27C). Reserve respiratory capacity, obtained by subtracting the maximum respiratory capacity from the routine oxygen consumption, is reduced as expected in HA-BOECs and reported to the basal level after correction (Figure 27D).

These results indicate a lower overall energy demand compared of the HA-BOECs compared to the healthy cells. Moreover, the reduction in electron transport chain capacity highlighted a direct defect in the complexes activity and confirmed the results obtained in the proteomic analysis.

FVIII enhances the proliferation of ECs

Considering our results showing impairment in the electron transport chain of HA-BOECs, we questioned if this alteration in ATP synthesis could lead to a different proliferation rate between C, HA and LV-FVIII HA-BOECs, analyzed by 7AAD assay. Curiously, we found no differences in G0/G1 and S phases and a slight but not significant increase of the G2/M phase in HA-BOECs compared to C and LV-FVIII HA-BOECs (Figure 28A). This result pointed out that maybe the absence of FVIII could induce a non-physiological iper-proliferation.

However, more importantly, we analyzed thanks to Incucyte, if both C and HA-BOECs respond to rhFVIII enhancing their rate of growth (Figure 28B). These results suggest that FVIII could act both in physiological and pathological systems as a growth factor.

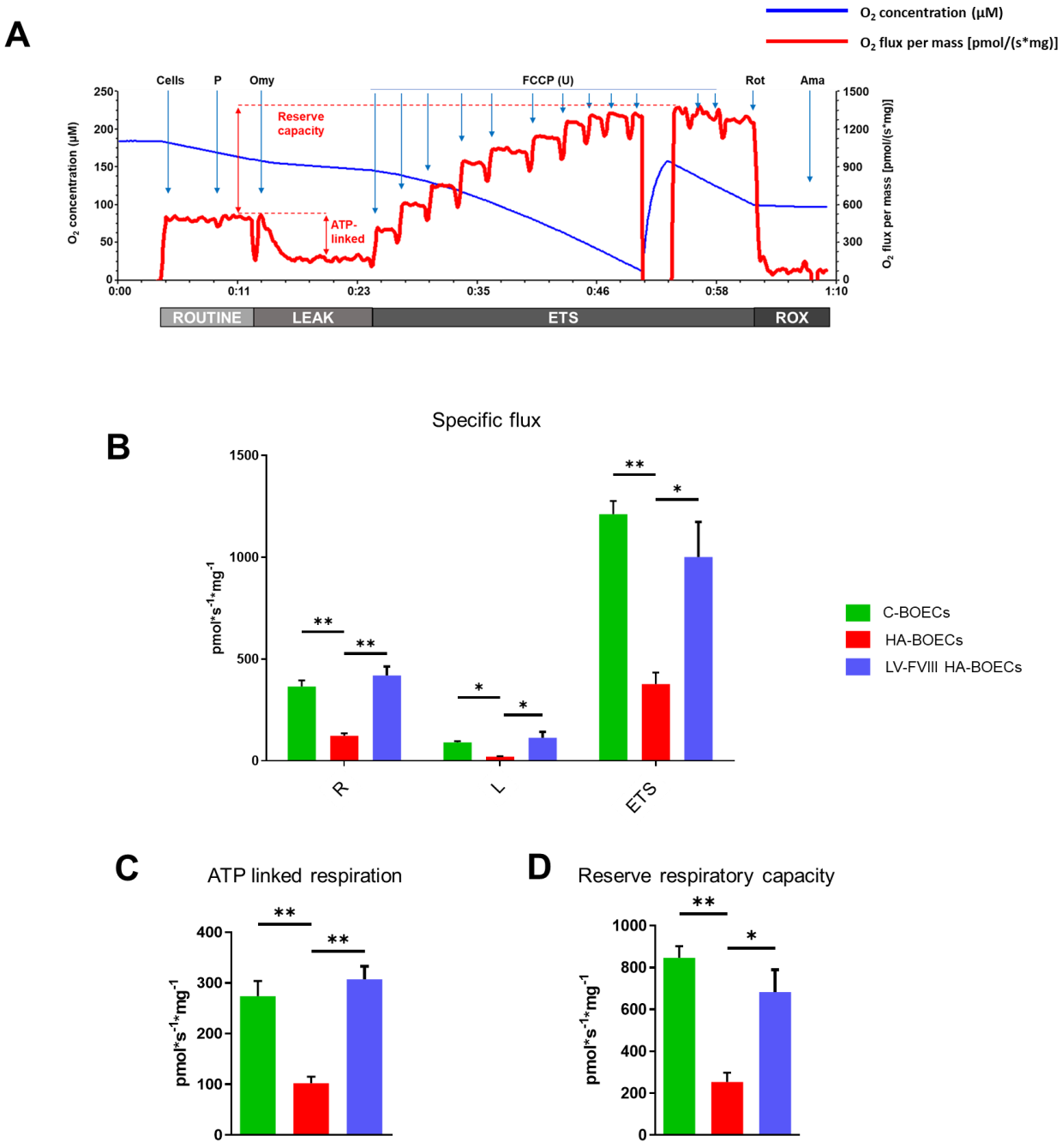


Figure 27. HA-BOECs have impaired mitochondrial respiration compared to C and LV-FVIII HA BOECs
A Representative oxygen consumption rate (OCR) of C2C12 myotubes after 24h treatment with 50 nM VDBP. **B** Mitochondrial respiration (specific oxygen flux in the routine state [R], leakage state [L], and maximal respiratory capacity [ETS]) in intact C2C12 myotubes was assessed for C, HA and LV-FVIII HA BOECs (n=3 for each group). **C** Oxygen consumption linked to ATP production, i.e., oligomycin-sensitive respiration obtained by the subtraction of L from R, for the samples as described in B. **D** Reserve respiratory capacity obtained by the subtraction of R from E, for the samples as described in B. Three biological samples for each group were analyzed in two independent experiment to obtained data shown in **B**, **C** and **D**. Data in **B**, **C**, and **D** are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA test (**p < 0.01; *p < 0.05).

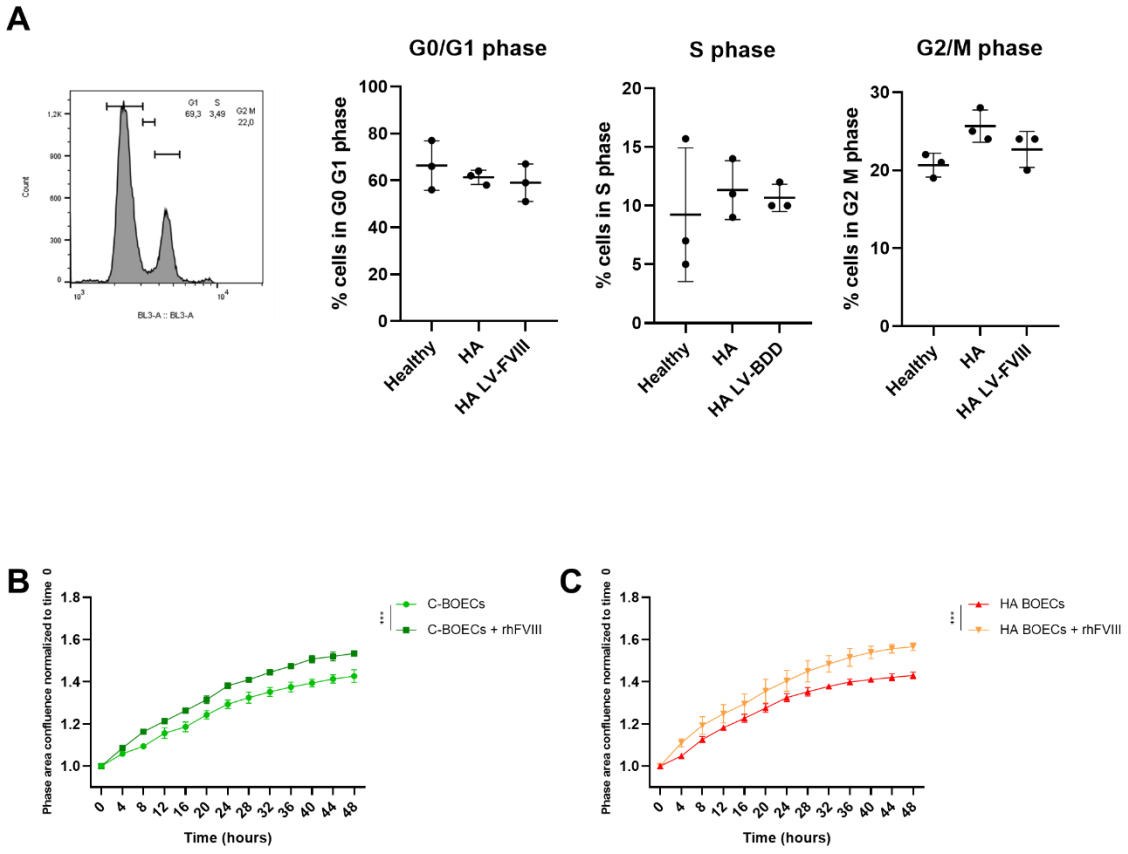


Figure 28. FVIII promotes proliferation in both C and HA BOECs
A Representative histogram obtained by 7AAD assay performed on C, HA and LV-FVIII HA-BOECs in which 3 phases can be distinguished: G0/G1, S and G2/M. The other three graphics represent the % of cells for each sample in each phase. **B** and **C** Representative curves of growth of C and HA BOECs respectively obtained with the artificial intelligence of Incucyte software analysis. Both kinds of cell were treated with rhFVIII (1UI/mL) which significantly enhances their proliferation. This experiment was performed three times for three different C-BOECs and three different HA-BOECs. Data in A, **B**, and **C** are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA test for **A** while two-way ANOVA was used for **B** and **C** (*** $p<0.001$).

Brain microvascular endothelial network of HA mice is impaired compared to WT mice

Many studies have reported that hemophilic patients suffer from intracranial hemorrhages (ICHs) but some of these works failed in differentiating between HA and hemophilia B (HB) patients (Zwagemaker et al. 2021, Zanon et al. 2022b). Thus, our investigation aimed to discern whether the absence of FVIII or FIX directly contributes to these bleeding incidents or if they arise from the hypocoagulable state shared by both forms of hemophilia.

Based on these data and our previous findings showing impaired functionality in HA ECs, we assessed histological sections of the brain microvascular endothelial network of 8 weeks old

WT, HA and HB mice (n=5 for each group) to verify possible differences caused by the deficiency of FVIII or FIX. To detect the vascular systems of the brains, we identified $CD31^+$ and claudin 5 $(CLDN5)^+$ cells. Interestingly, we found that the vascular density of HA brains (Figure 29B) resulted drastically reduced compared to WT brains (Figure 29A) corroborating our hypothesis that HA ECs are impaired compared to WT ECs while no difference between WT and HB brain vascular system (Figure 29C) has been assessed (Figure 29D). These data suggest that the lack of FVIII but not FIX impairs brain endothelial network highlighting the role of FVIII in the maintenance of a correct EC functionality.

To explore whether this impairment is present also in younger and older mice, we performed the same immunofluorescent analysis on brain sections taken from 3 weeks (Figure 30A) and 24 weeks old mice (Figure 30C). Similar results were obtained in these experiments compared to 8 weeks old mice highlighting that the vascular network of HA mice is significantly reduced compared to WT ones independently to the age of the mice (Figure 30B, D). Therefore, we injected HA mice with LV-FVIII and these mice reached up to 10% of FVIII activity after 16 weeks post LV injection. Thus, when they were 24 weeks old, we evaluated their brain vascular structure comparing them with WT and HA mice of the same age. Surprisingly, we observed that LV-FVIII mice showed a brain vascular density similar to WT mice (Figure 30D, E).

To further investigate the relation between FVIII and brain ECs, we optimized a protocol (see Materials and Methods) to successfully isolate brain microvascular endothelial cells (BMECs) from both WT and HA murine brain. After isolation, we performed FACS analysis to verify the percentage of $CD31^+$ (endothelial cells, in blue, around 70% for both groups) and $CD45^+$ cells (microglia, in orange, around 30%) in our sample (Figure 31A). We cultured in collagen-coated wells and both WT and HA BMECs grew similarly reaching 80% confluence in 4 days (Figure 31B, C). The used medium is specific for ECs killing $CD45^+$ cells. Indeed, immunofluorescence staining shows the cultured cells are >98% $CD31^+/CLDN5^+$ and no $CD45^+$ cells persist in the plate (Figure 31D). Only a small percentage of pericytes ($alphaSMA^+$) simultaneously grow with BMECs (Figure 31D).

Taken together, these data show a drastic reduction of brain microvascular endothelial network in HA mice compared to WT and, crucially, highlight the importance of FVIII re-introduction in HA mice to restore not only the correct hemostasis system but also a physiological vascular network. Moreover, the optimized protocol for the BMEC isolation will allow to study in depth

possible functional and transcriptomic differences of these cells to gain further insights about the role of FVIII in brain ECs.

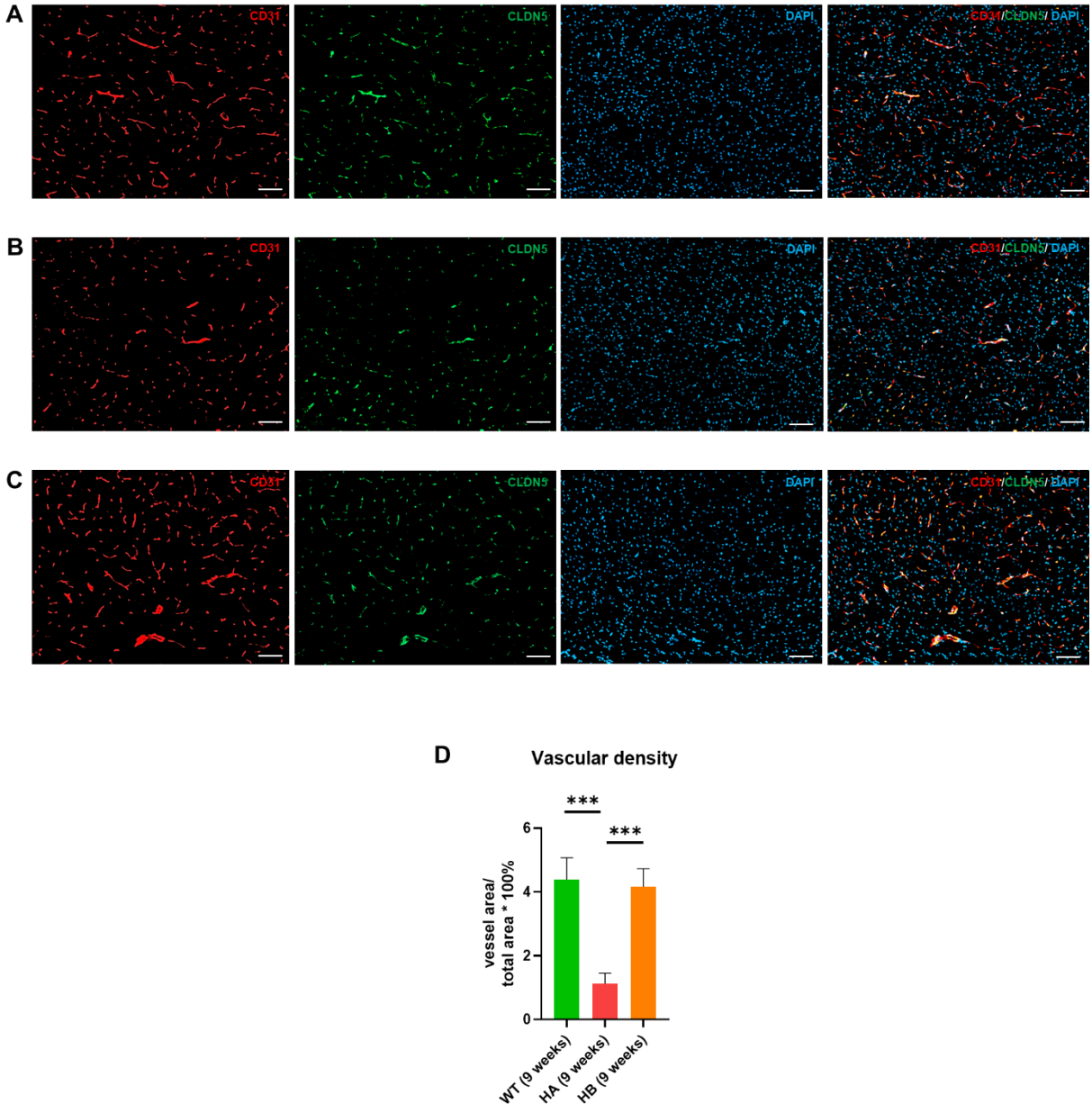


Figure 29. Brain vascular network of adult HA but not HB mice is impaired compared to WT mice
A Representative immunofluorescence of histological section of a brain taken from 8 weeks old WT mouse and stained for CD31 (first panel, red), CLDN5 (second panel, green), and DAPI (third panel, blue). The last panel represent the merge image. Scale bar= 200µm **B** Same as A for a brain taken from 8 weeks old WT mouse. **C** Same as A for a brain taken from 8 weeks old hemophilic B (HB) mouse. **D** Quantification of vascular density of WT, HA, and HB mice (n=5 biological samples and at least 3 slices per brain were assessed). Data in D are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA test (*** $p<0.001$).

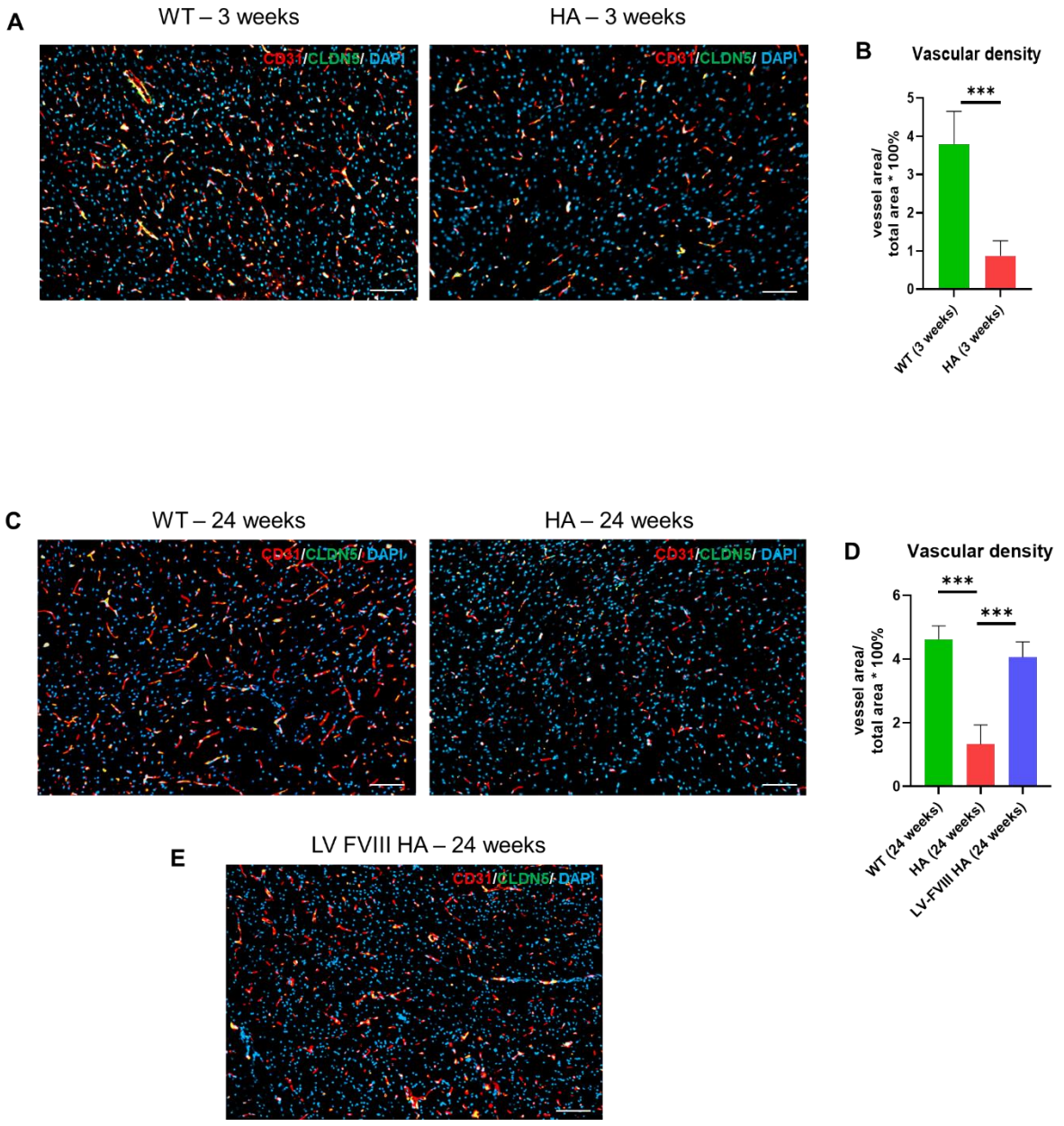


Figure 30. FVIII is essential for brain vascular network development and maintenance
A Representative immunofluorescences of histological sections of brains taken from 3 weeks old WT and HA mice and stained for CD31 (red), CLDN5 (green), and DAPI (blue). The image represent the merge of all the colors. **B** Quantification of vascular density of 3 weeks old WT and HA mice (n=5 biological samples and at least 3 slices per brain were assessed). Scale bar= 200µm **C** Same as A for brains taken from 24 weeks old WT and HA mice. **D** Same as B for a brain taken from 24 weeks old WT, HA and LV-FVIII HA mice (n=4 biological samples and at least 3 slices per brain were assessed). **E** Same as A for brains taken from 24 weeks old LV-FVIII HA mice. These mice were injected with LV-FVIII when they were 8 weeks old. Data in **B**, and **D** are expressed as mean ± SD. Statistical analysis was performed by one-way ANOVA test (*** $p<0.001$).

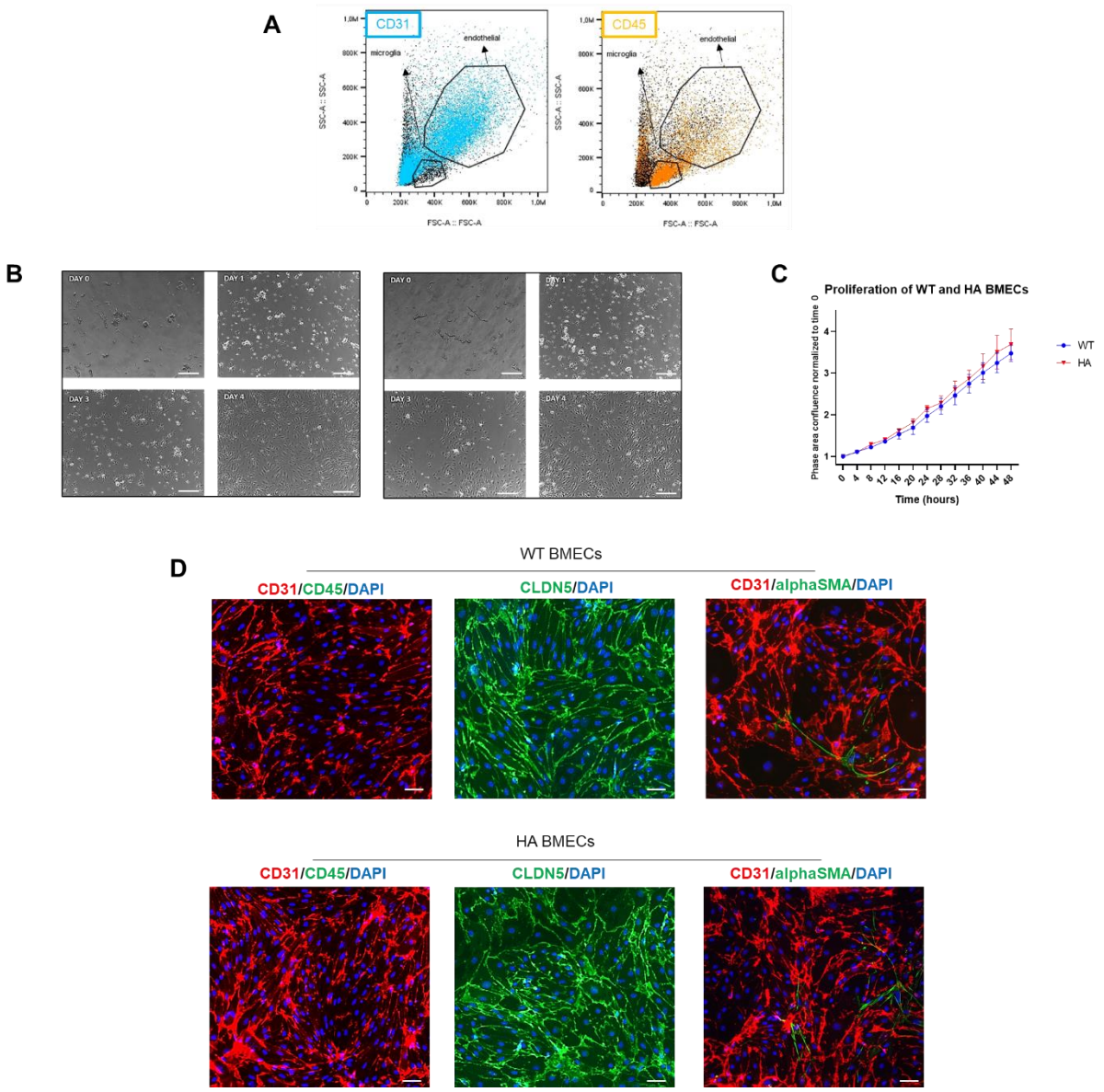


Figure 31. Isolation and characterization of WT and HA BMECs
A Representative dot blot from flow cytometry analysis showing the cells isolated after the brain disaggregation and digestion. In blue (first panel) are evidenced the $CD31^+$ cells both in the upper right part (live cells, gate called "endothelial") and in the lower left corner (debris or dead cells). In orange (second panel) are evidenced the $CD45^+$ cells mainly clustered in a gate named "microglia". **B** Representative images of brain microvascular endothelial cells (BMECs) obtained from WT and HA mice and followed in culture for 4 days (n=6). Scale bar=200µm. **C** Representative curves of growth of WT and HA BMECs respectively obtained with the artificial intelligence of Incucyte software analysis. **D** Immunofluorescence for CD31, CLDN5, CD45 and alphaSMA on BMECs obtained from WT and HA mice after 4 days of culture. Scale bar= 200µm. Data in **C** are expressed as mean ± SD.

Discussion

FVIII is a plasma protein essential for blood clotting as its mutations lead to HA, a recessive X-linked bleeding disorder caused by coagulation defects (Castaman and Matino 2019). Currently, no definitive cure exists for HA, with replacement therapy utilizing rFVIII as the conventional treatment method. This treatment consists in intravenous injections as needed to address acute hemorrhages or adhering to prophylactic schedules to reduce bleeding episodes (Collins et al. 2009, Franchini and Liumbruno 2019). However, despite considerable improvements, replacement therapy still faces a significant limitation, as approximately 5-30% of HA patients who receive FVIII develop neutralizing antibodies (inhibitors) against it, rendering the treatment ineffective. To address this challenge, a novel class of molecules known as non-replacement therapies has been introduced for the treatment of HA. One such therapy is Emicizumab, a monoclonal antibody with bispecific activity that mimics the co-factorial role of FVIII by bridging activated FIX and FX (Kitazawa et al. 2012). Some studies conducted with Emicizumab have revealed great advantages in restoring physiological coagulation even if in some cases, the bleeding episodes persist despite the treatment with Emicizumab (Zimowski et al. 2019, Glonnegger et al. 2022, Batsuli et al. 2023, Garcia et al. 2023, Pasca et al. 2023).

Generalized bleeding is a hallmark of HA, affecting various areas of the body, with joints and muscles being particularly susceptible sites. In addition to bleeding, HA is associated with various pathological conditions such as arthropathy, bone remodeling issues, renal and cardiovascular diseases, and intracranial hemorrhages. Recent clinical and pre-clinical findings have revealed that HA individuals may encounter vascular issues. Some studies have pointed out possible endothelial dysfunction in HA patients, as indicated by reduced flow-mediated dilation (FMD) and decreased hyperemic velocity time integral (VTI) when compared to healthy individuals (Sartori et al. 2008, Biere-Rafi et al. 2012, Sun et al. 2017). Such evidence highlights the importance of closely monitoring and addressing vascular problems in HA patients to ensure their overall well-being. These results indicate significant alterations in both macrovascular and microvascular endothelial functions. Yet, the role of FVIII in EC homeostasis and functionality remains largely unexplored. While some research has investigated the extra-coagulative effects of FVIII in bone remodeling and macrophage polarization, more studies are required to fully comprehend the implications of FVIII in endothelial function (Cadé et al. 2022a).

In this study, we demonstrate that FVIII has a significant regulatory role in EC functionality beyond its well-known function in blood clotting. It is worth noting that in previous research, we have successfully transduced BOECs obtained from both healthy volunteers and patients with severe HA with LV carrying FVIII and, once implanted into a prevascularized medical device or in association with microcarrier beads, these cells have been shown to correct the bleeding phenotype in HA mice (Olgasi et al. 2021). Now, our comprehensive analysis reveals that HA-BOECs exhibit defects in *in vitro* angiogenic tests, along with *in vivo* increased vessel permeability. These dysfunctions are corrected by LV transduction of FVIII or by rFVIII treatment, supporting the hypothesis that FVIII contributes to vascular stability. Our results also indicate that the beneficial role of FVIII in EC functionality is independent of its quantitative expression level. Indeed, treatment of C-BOECs with rFVIII did not improve their functionality, suggesting that endogenous FVIII expression is sufficient to maintain a correct endothelial phenotype. Correspondingly, LV-FVIII HA-BOECs, expressing higher-than-normal levels of FVIII, displayed *in vitro* behavior similar to that of C-BOECs. These data were corroborated by knocking out *F8* in healthy ECs. KO-*F8* ECs show a significant impairment in all functions and rFVIII acute treatment was able to partially restore their functionality.

It is possible to speculate that FVIII plays a crucial role in maintaining the integrity of junctional proteins or extracellular matrix composition, thereby enhancing the stability of ECs. This hypothesis is supported by previous studies that have shown an increased synovial vascular permeability post-joint injury in HA mice, which highlights the vulnerability of the vasculature in the absence of FVIII (Bhat et al. 2015, Cooke et al. 2018, 2019, Gopal et al. 2021). Vascular permeability is essential for facilitating the exchange between blood vessels, tissues, and organs, playing a crucial role in maintaining physiological balance. However, an excessive permeability could be the cause of general spontaneous bleeding episodes, present in HA patients. Indeed, increased vascular permeability is one of the major indicators of the blood vessel damage and the loss of EC integrity. The modulation of vascular permeability is the result of an intricate relation between several mediators (such as prostaglandins and prostacyclin, nitric oxide, vascular growth factor, and cytokines) and membrane receptors (including vascular cell adhesion molecule (VCAM), intercellular cell adhesion molecule (ICAM), vascular endothelial growth factor receptor (VEGFR-2), receptor for advanced glycation end products (RAGE) (Wautier and Wautier 2022).

For these reasons, our research went deeper to understand if FVIII is able to modulate EC function binding specific membrane receptor(s). Over the past decades, a deeper understanding of vascular EC functions and their interactions with plasmatic proteins has emerged. In particular, many coagulation factors (CFs) have been described to control EC functions, including angiogenesis, migration, proliferation and, especially, permeability (Olgasi et al. 2024). Several CFs have undergone extensive research and are known to bind specific receptors and trigger intracellular signaling pathways that affect EC functions. However, for others, the receptors and the triggered molecular mechanisms have not been fully clarified. Nonetheless, emerging evidence suggests their ability to elicit a response from ECs. That is why, recent studies have been investigating the relation between FVIII and EC functionality. In particular, one research demonstrated that FVIII can modulate both transcriptional and functional alterations in ECs, resulting in reduced adherence and increasing permeability *in vitro*, with paxillin identified as a key mediator of these effects (Cadé et al. 2022b). However, these results show some limitations: i) they used human umbilical venous ECs (HUVECs), where FVIII in not universally recognized to be expressed (Shovlin et al. 2010b); ii) molecular mechanism triggered by FVIII in ECs was not fully illustrated; iii) the possible receptor of FVIII was not identified. The authors speculate that lipoprotein receptor related protein 1 (LRP-1), a FVIII scavenger receptor, could bind FVIII triggering a pathway modulating EC functionality. However, many receptors control the bioavailability of FVIII including low density lipoprotein receptor, stabilin-2, C-type lectin domain family 4 member M, asialoglycoprotein receptor and scavenger receptor class A member 5, but they have never been described to induce an intracellular signaling able to modify EC functionality after FVIII binding (Figure 7) (Cadé et al. 2022a).

Thus, to gain deeper mechanistic insights into the role of FVIII, we explored possible receptors and pathways activated by FVIII in ECs. We found that FVIII binds to ITGB1, triggering the FAK/Src signaling pathway, crucial for modulating angiogenesis, migration, and permeability in these cells (Figure 32). This binding is not a surprise considering that many CFs exert their role on EC homeostasis binding G-protein coupled receptors (GPCRs), receptor tyrosine kinases (RTKs), and especially integrins (Figure 7) (Olgasi et al. 2024).

Building on these findings, transcriptomic analysis performed on BOECs from healthy controls and HA patients uncovered significant differences ascribable to FVIII expression. Of note, many

genes and proteins downregulated in HA-BOECs were restored following LV-FVIII reintroduction. Accordingly, GSEA and GO analysis of differentially expressed genes and proteins, identified pathways corresponding to vascular development, cell adhesion and migration, and ECM organization, suggesting that the endothelial dysfunctions observed in our *in vitro* and *in vivo* experiments could likely be associated with diminished ECM protein expression in HA ECs. Indeed, ECM plays a critical role in maintaining the structure of vascular ECs in blood vessels, regulating EC migration, angiogenesis, and the formation of new blood vessels (Bischoff et al. 2005, Olgasi et al. 2024). This altered ECM profile in severe hemophilic patients might therefore be a contributing factor to their spontaneous bleeding episodes. Our results are supported by several studies describing a relation between matrix component turnover, especially collagens, and patients annual bleeding rate in HA patients (Kjeld et al. 2018b). A recent study also showed elevated collagen IV and VIII markers in the plasma of HA patients (Manon-Jensen et al. 2024b), suggesting an impairment of the ECM metabolism and stability.

To confirm whether FVIII-induced signaling affects ECM gene expression, we focused on NID2, a cell adhesion glycoprotein, which was markedly downregulated in HA-BOECs compared to C-BOECs but fully restored in LV-FVIII HA-BOECs. Our data show that NID2 expression is contingent upon FAK activity, as evidenced by the inability of FVIII to stimulate NID2 expression in HA-BOECs treated with defactinib, a compound that blocks FAK phosphorylation (Figure 32). Importantly, the failure of rFVIII treatment to reverse dysfunction in NID2-deficient ECs demonstrates the requirement of NID2 for FVIII-mediated regulation of EC processes. Existing research indicates that NID2 is crucial for endothelial basement membrane integrity largely due to its interaction with collagen IV, perlecan - modulated in our transcriptomic and proteomic analysis too – and laminins (Töpfer and Holz 2024). Recent studies have further clarified the role of NID2 in regulating the EC phenotype, revealing its significant impact on organizing the ECM and modulating intracellular pathways (Bader et al. 2005, Pozzi et al. 2017, Mao et al. 2021, Chen et al. 2022). Perlecan and NID2 contribute to structural integrity by linking collagen IV and laminin networks, thereby enhancing overall stability. The observed reduction in NID2 expression in the basement membrane of ECs isolated from severe HA patients may thus lead to impaired collagen IV and laminin network formation and a general increased vessel fragility.

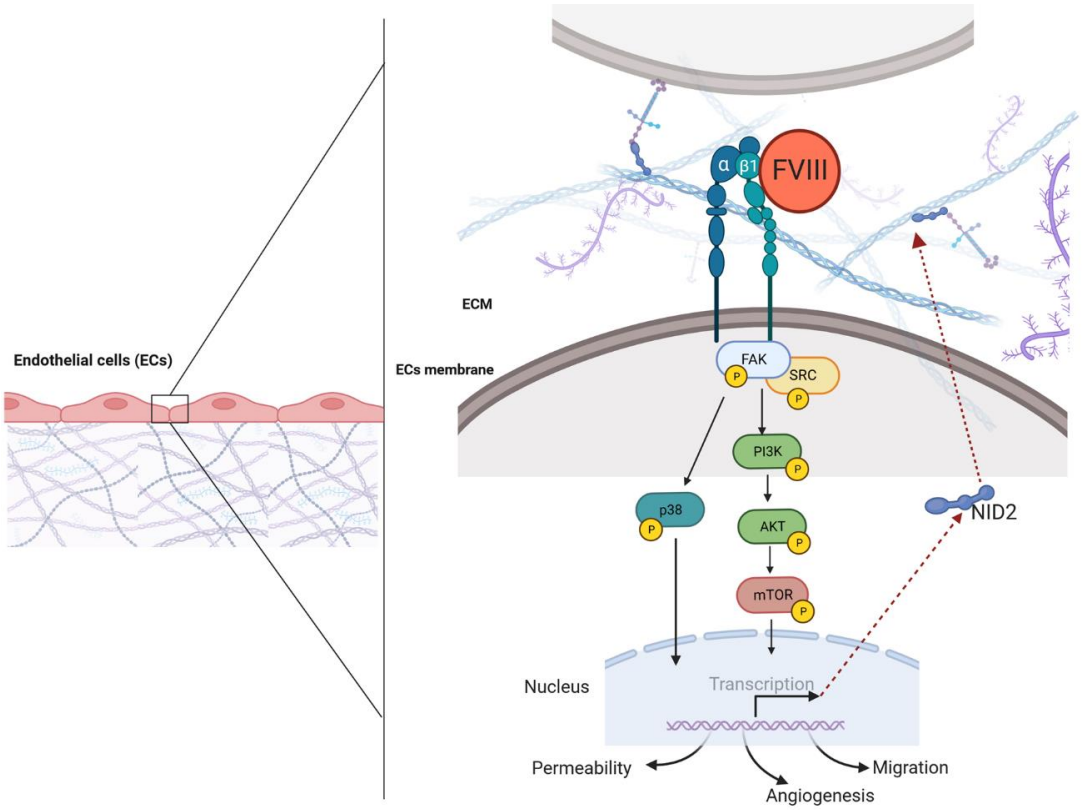


Figure 32. FVIII-ITGB1 signaling pathway
This schematic, created using BioRender, depicts the signal transduction pathways activated by the interaction of FVIII with ITGB1. According to our model, FVIII binds to ITGB1 on the endothelial cell (EC) surface, as demonstrated by co-immunoprecipitation experiments. This binding triggers an intracellular signaling cascade involving the phosphorylation of FAK/Src, subsequently activating downstream targets such as AKT, mTOR, and p38 MAP kinase. The signaling extends to the nucleus, prompting transcriptional responses that regulate angiogenesis, migration, and permeability. Among the genes induced by this pathway, we identified NID2 as a critical molecule in the EC response to FVIII.

In addition to the analysis of the transcriptomic profiles, we performed proteomic analysis that not only confirmed transcriptomic data but also revealed new protein sets modulated by FVIII. Among them, the most significant modulated cluster is the one related to cellular respiration and mitochondrial activity. The reduced expression of mitochondrial proteins was reflected also in the functional assay showing a lower overall energy demand of HA BOECs compared to healthy ones. Indeed, the application of OROBOROS respirometry provided an extensive analysis of the respiration patterns of C, HA and LV-FVIII HA-BOECs, allowing for a more profound insight into their mitochondrial activity. The mitochondrial electron transport chain (ETC) serves as a crucial mechanism for meeting the energy demands of the cell, playing a central role in cellular respiration. Importantly, the mitochondrial ETC not only generates adenosine triphosphate (ATP) but also helps to maintain cellular homeostasis by regulating

reactive oxygen species (ROS) production and participating in various cellular signaling pathways. Alterations of this cellular mechanism compromise the response of the cells to various external stimuli, including cellular stress (Zhao et al. 2019). Thus, considering our data, we can speculate that the impaired functionality of HA-BOECs and their lower expression of pivotal ECM proteins may be also caused by their altered metabolism. Deeper studies are needed to confirm this hypothesis and a direct link between FVIII and mitochondrial functionality needs to be elucidated. It will be interesting to understand if the altered metabolism described in HA-BOECs can be directly caused by the reduced expression of SOD2. Indeed, this protein has been widely recognized to regulate oxidative stress levels within the mitochondria, thereby preserving mitochondrial integrity and preventing cellular damage. Dysregulation of SOD2 has been implicated in various pathological conditions, including neurodegenerative diseases, cardiovascular disorders, and cancer, highlighting its significance in maintaining cellular health and homeostasis (Palma et al. 2020). Moreover, alterations of mitochondrial ETC have been usually found related to altered proliferation but no statistical differences in any phase of the cell cycle were revealed by our analysis. However, we found that FVIII was able to enhance the proliferation of both C and HA-BOECs suggesting that it could also play as a growth factor for ECs. Considering these previous data which strongly pointed out the pivotal role of FVIII in EC homeostasis and functionality, we subsequently aimed to evaluate the endothelial network structure in WT and HA mice to verify if the absence of FVIII may impair the *in vivo* vasculature. The brain was selected as the initial organ of the study, as we hypothesized that intracranial hemorrhages (ICHs) in HA patients could stem from fragility in the brain vasculature. Our analysis indeed revealed a significant reduction in the vascular network of the brains of HA mice compared to WT mice. Importantly, in vivo gene therapy was able to restore a phenotype akin to WT mice. It is noteworthy that brains from hemophilia B (HB) mice exhibited similar vascular density to WT mice, indicating that the absence of FIX does not compromise brain vasculature. This underscores the direct role of FVIII in maintaining brain ECs. These preliminary data pave the way for new fascinating research about how FVIII influences the proliferation, development, and maintenance of brain vasculature. Due to the limited number of assessed brains (at least 4 per group), these same histological analyses need to be performed again to confirm this result. Moreover, future studies will focus on the functional assay to assess the permeability of the blood barrier brain (BBB) of

WT, HA and LV-FVIII HA mice. Increased BBB permeability has been implicated in various pathologies, including diabetes and coagulopathies, and understanding its molecular mechanisms is crucial for preventing potential brain hemorrhagic events (Xu et al. 2017, Kim et al. 2020, Zhang et al. 2020, Tsuneoka et al. 2021). These future results could strongly reinforce our hypothesis that ICHs are caused by a functional impairment of the brain vasculature. Finally, the vascular network structure and functionality of other organs of HA mice need to be characterized, starting from the microvasculature of the liver, which mainly produces FVIII in the body, and then, the kidney and the heart. These data will help in understanding if the renal and cardiovascular complications typical of HA patients could be caused, at least in part, by an altered vascular functionality.

It is important to note that this study is an initial proof of concept with solid data but which needs to be further developed and it shows some limitations. The first limitation consists of the use of BOECs obtained from a limited number of subjects. Indeed, for the functional assays C-BOECs from 5 healthy volunteers and HA-BOEC from 4 HA patients were used. Most of the assays were performed between passages 4 and 8. Using these cells with higher passages should be avoided due to several reasons related to changes in cellular characteristics and functionality over time. New isolation of BOECs from both healthy subjects and HA patients will be carried out to confirm these data. Moreover, to determine if only FVIII and not FIX plays a crucial role in vascular maintenance and development, BOECs from HB patients should be isolated and challenged in the same functional assays. Another limitation of this study is the lack of identification of the integrin alpha chain. Indeed, an integrin molecule is composed of two noncovalently associated glycoproteins, α and β. For the first time, we were able to identify the binding between FVIII and ITGB1, which is the most versatile beta chain able to bind up to 12 alpha chains. However, further studies are needed to detect which alpha chain is necessary to trigger the signaling pathway that we identified. It is important to note that ITGA5 is one of the most described ITGB1 binding partner in ECs and, for this reason, our future studies will firstly focus on that. Finally, the pathway that we have described could be not complete and other intracellular actors may be responsible for the complex regulation of ECs triggered by FVIII. Surely, it is interesting to demonstrate if p38 and mTOR are the only actors which go to the nucleus, after FAK activation, and stimulate NID2 (and possibly other ECM proteins) expression.

In conclusion, our research suggests that FVIII deficiency in HA ECs leads to impairments beyond coagulation, specifically affecting endothelial signaling and predisposing HA ECs to increased vessel fragility and altered permeability. Therefore, FVIII emerges not merely as a coagulation factor but also as a critical regulator of angiogenesis and a promoter of endothelial barrier stability, providing fresh insights into vascular physiopathology. Further investigations conducted on more advanced EC models (e.g. organ-on-a-chip) and on preclinical models of HA would help in understanding how the FVIII can impact the ECs development, functions and maintenance within tissues and organs during embryogenesis and in adult life. From a clinical perspective, these findings suggest that FVIII replacement therapy in HA patients could offer benefits that go beyond improved clotting, such as enhanced vascular health and resilience. This new perspective on FVIII paves the way for future research aimed to develop more holistic treatment strategies for HA, targeting both coagulation deficiencies and vascular stability.

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