



UNIVERSITÀ DEL PIEMONTE ORIENTALE

School of Medicine
Department of Health Sciences
Master's degree in Medical Biotechnology

Master Thesis

The study of the vascular system in the brain of
Hemophilia A mice

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Data: 30.06.2025 19:20:04 CEST
Organizzazione: UNIVERSITA' DEGLI
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Data: 30.06.2025 15:27:24 CEST
Organizzazione: UNIVERSITA' DEGLI
STUDI DEL PIEMONTE
ORIENTALE/01943490027

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Academic year 2024/2025

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Abstract

Haemophilia A (HA) is a congenital bleeding disorder caused by mutations in the factor 8 (F8) gene which result in reduced levels and/or activity of Factor VIII (FVIII). HA occurs in approximately 1:5000 male newborns and it is classified based on residual FVIII activity: severe (<1%), moderate (1–5%), and mild (5–40%). Clinically, HA is characterized by spontaneous or trauma-induced bleeding episodes that can occur in any part of the body. Among the possible complications—such as hemarthrosis, renal and cardiovascular disease, the most serious clinical manifestation of haemophiliac patients is intracranial haemorrhages (ICHs), both in neonates and the elderly. Although the exact mechanisms underlying cerebral hemorrhages in HA remain poorly understood, several studies have demonstrated a correlation between HA severity and increased ICH risk. Emerging evidence suggests that beyond its coagulation function, FVIII may play a broader role in maintaining endothelial cell (EC) homeostasis. Notably, ECs derived from HA patients exhibit reduced functionality compared to those from healthy individuals, raising the possibility that cerebral endothelial dysfunction may contribute to the pathogenesis of ICH in HA. This study aims to explore this hypothesis by investigating structural and functional alterations in cerebral vasculature and blood-brain barrier (BBB) in a murine model of HA compared to WT. HA and WT mice of 3, 8, 24 and 52 weeks of age were examined. Histological brain sections were prepared and subjected to immunofluorescence to visualize and quantify blood vessels using fluorescence microscopy. The results show a significant reduction in vessel density and vessel length in HA brains compared to WT. Moreover, brain microcirculation endothelial cells (BMECs) were successfully isolated to study directly the cellular components of the BBB. Following confirmation of endothelial identity through phenotypic characterization, functional assays including proliferation and migration were performed. Findings indicate that FVIII deficiency is associated not only with compromised vascular architecture but also with impaired endothelial cell function, specifically reduced proliferative and migratory capacity can cause, not only an altered structural function of the vessels, but also be associated with incorrect cell proliferation and migration. These data support the hypothesis that FVIII plays a critical extracoagulant role in cerebral vascular integrity and BBB function, offering new insight into the vascular complications seen in HA.

Introduction

Hemophilia A

Hemophilia A is a congenital coagulopathy caused by the lack or insufficiency of FVIII ¹. FVIII is a glycoprotein produced primarily by the liver sinusoid endothelial cells (LSECs) and released into the bloodstream. The F8 gene on the long arm of the X chromosome (Xq28), has a length of 182 kb and is composed of 26 exons. Mutations in this gene, whether inherited or occurring de novo, are diverse and lead to a decreased activity of Factor VIII (FVIII) ². As the gene is located on the X chromosome, hemophiliacs are mainly male (1 in 5-10. 000 males), while female subjects are healthy carriers ³. Females usually carry the defective gene without showing symptoms, although in rare situations, such as skewed X-chromosome inactivation or the presence of mutations on both alleles, they may exhibit mild bleeding tendencies. Several mutations can characterize different degrees of severity of the disease. In fact, three clinical conditions can be described: a form of mild HA, which occurs when the residual activity of FVIII varies from 5% to 40% and is generally diagnosed in adulthood. A moderate form of HA, which occurs when the FVIII varies from 1% to 5%, so those affected have moderate bleeding in particular and after trauma of low and medium extent or after surgery. The severe form, where FVIII activity is less than 1%, is often diagnosed at birth or within the first two years of life and is characterized by frequent bleeding, in particular at the level of the skull and patients may present frequent subcutaneous hematomas due to spontaneous bleeding ⁴. Spontaneous bleeding events are predominant in patients with severe HA, often leading to early diagnosis, while moderate and mild forms are generally associated with bleeding following trauma or surgical interventions ^{5,6}. Although the majority of HA cases are inherited, around one-third occur spontaneously, caused by de novo mutations in the F8 gene. These cases arise without a known family history, which underscores the importance of genetic screening and counseling in new diagnoses ⁷. The clinical manifestations of hemophilia patients are mainly spontaneous or trauma-induced bleeding in different parts of the body depending on the severity of the disease. The blood vessel, or joint bleeding, is the haemorrhagic manifestation of severe hemophilia ⁸. These hemorrhagic events usually begin when the subject begins to walk in his early years. A severe decrease in FVIII can compromise the integrity of joints, causing damage to cartilage, synovial tissue and bone, resulting in the following skeletal muscle problems, including elevated risk of fractures and osteoporosis ⁹. Hemophiliac patients may also

develop cardiovascular and kidney disease, mainly caused by hypertension, that has been detected with an increased prevalence in hemophilia subjects ¹⁰.

Structure and function of coagulation factor VIII

FVIII is a glycoprotein, crucial in blood coagulation cascade, encoded by the homonymous gene (F8) present on the long arm of the X chromosome (Xq28). The gene is 182 kb in length and it is composed of 26 exons, with exon 14 that is the largest and that encode for the B domain of the protein, and 25 introns. The F8 gene transcribes for an mRNA of about 9 kb in length, which produces an inactive precursor protein of 2332 amino acids with a molecular weight of about 263 kDa. FVIII is composed of six structural domains: A1, A2, B, A3, C1 and C2 ^{11 12 13}. Once synthesized, FVIII is cut by a protease called furin into two chains: the heavy chain of 200 kDa weight is formed by domains A1, A2 and B, and a light chain of 80 kDa consisting of A3, C1 and C2. FVIII is activated by thrombin, which cleaves the protein at residues Arg372, Arg740 and Arg1689, resulting in the removal of the B domain and in the formation of activated FVIII (aFVIII), a heterotrimer composed of the A1, A2, A3, C1, and C2 domains. aFVIII binds to the phospholipid surface (PS) through C1 and C2 domains ^{14,15} and initiates its effect on activated factor IX (FIXa) within a complex that triggers the activation of factor X (FX) ^{16,17} aFVIII binds FIXa forming the tenase complex and to FX ^{18,19}. 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 ^{20,21}. 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 ^{13,22}.

Endothelial tissue

The endothelium is the tissue that lines the inner surface of blood vessels, lymphatic vessels, and the heart. It is composed of a single layer of squamous cells, called endothelial cells, which on the luminal side are in direct contact with the blood, while on the basal side are anchored to the basal lamina, forming the tunica intima of the vessel,

which is in turn connected to the underlying tissues such as the tunica media and adventitia²³. Structurally, the endothelium is similar among various organs, while it differs functionally depending on its location²⁴. According to stimuli, it is able to secrete numerous molecules, with autocrine and paracrine action, capable of modifying its own behaviour and that of neighbouring cells; this results in modulation of vascular tone and blood flow. The main molecules released have either a pro- or anticoagulant action. The most important anticoagulant mediators are: tPA (tissue plasminogen activator), which activates the transformation of plasminogen into plasmin; prostacyclin PGI₂, which causes vasodilation and prevents platelet aggregation; heparin-like glycosaminoglycans, which increase antithrombin activity; thrombomodulin, which is a cofactor of thrombin; and nitric oxide (NO), which is a powerful vasodilator and inhibitor of platelet activity. Regarding the procoagulant proteins released, we find FVIII, von Willebrand factor (vWF), which adheres to the vessel wall following endothelial injury and allows platelet adhesion to collagen, activating their aggregation; tissue factor (or tissue thromboplastin), which activates coagulation factor VII; and endothelin, which increase the vasoconstrictor activity of hormones, promote platelet aggregation and leukocyte activation²⁵. The endothelium plays a crucial role in maintaining the structure and function of the vascular wall by regulating permeability and vascular tone, coagulation, fibrinolysis, and mediating the inflammatory response. Among the various functions, endothelial cells are also responsible for the formation of blood vessels, a process called angiogenesis²⁶. This process occurs through the sprouting of new vessels from pre-existing ones, stimulated by specific pro-angiogenic signals including members of the vascular endothelial growth factor (VEGF) family, angiopoietins, transforming growth factors, platelet-derived growth factor, tumor necrosis factor alpha, interleukins, and members of the fibroblast growth factor family. In particular, VEGF is the most important and well-known proangiogenic protein described so far. In fact, VEGF is able to induce proliferation, sprouting, and tube formation of endothelial cells²⁷. Among the various organs where endothelial cells form vessels are present, a crucial role is played in the central nervous system, where endothelial cells are specialized to form the blood–brain barrier (BBB)²⁸, a protective structure for brain tissue against potentially harmful elements present in the blood, while allowing the passage of necessary substances to the brain. Endothelial cells, particularly in this system at the capillary level, are distributed to form a continuous endothelium, through junctional complexes classified as: adherens junctions, tight junctions, and gap junctions²⁹.

Endothelial fragility in hemophilia patients

Several studies have observed impaired endothelial function in hemophilia patients, particularly at the microcirculatory level, along with altered levels of specific collagen types in the plasma, suggesting endothelial dysfunction³⁰. Notably, elevated levels of collagen XVIII have been detected in the plasma of hemophilia A (HA) patients and were found to correlate with higher annual bleeding rates^{30,31}. It is important to emphasize that the extracellular matrix (ECM)—of which collagens are key components—plays a crucial role in tissue homeostasis. In various pathological conditions, including bleeding disorders, abnormalities in ECM protein synthesis and degradation have been documented³². The first hypothesis of endothelial dysfunction in hemophilic patients was proposed in a 2008 study, which reported reduced arterial dilation in these patients compared to healthy controls, as measured by a technique known as flow-mediated dilation (FMD)³³. However, these findings were not confirmed by a subsequent study in 2017, which showed comparable arterial dilation between hemophilia patients and healthy volunteers. Instead, that study demonstrated that endothelial dysfunction in hemophilic patients is predominantly present at the microvascular level³⁴. Additionally, altered and uncontrolled vascular remodelling has been observed in the joints of hemophilic mice following the induction of hemarthrosis³⁵. This phenomenon was further supported by findings from two different hemophilic mouse models, which exhibited increased vascular permeability following joint injury³⁶. Taken together, these findings suggest that ECM and endothelial dysfunction may contribute to increased vascular permeability in hemophilic patients, potentially underlying the frequent bleeding episodes. Nevertheless, it remains unclear whether FVIII replacement therapy has any significant impact on neo-angiogenesis. Further studies are needed to explore the potential extra-coagulative role of FVIII on endothelial biology and to determine whether prophylactic treatment plays a crucial role in maintaining proper endothelial and vascular homeostasis in hemophilic patients.

The role of Endothelial Cells and factor VIII in vascular integrity and hemophilia A

Endothelial (EC) cells play the key role of lining the lumen of the vascular system and contribute to the maintenance of vascular homeostasis, regulate blood flow, participate in the coagulation process and contribute to the modulation of immune responses in various tissues. The ECs are not all the same, but they differ in various subtypes according to

tissue and function. With regard to the liver, it has been shown that LSECs are the main FVIII producers on a physiological level^{37,38}. In addition to LSECs, there are other endothelial subtypes that contribute to the production of FVIII, including blood outgrowth endothelial cells (BOECs). BOECs are a subset of adult human endothelial cells that can be isolated from peripheral blood, in particular derived from peripheral mononuclear blood cells and cultured under pro-endothelial conditions. BOECs maintain a fully differentiated endothelial phenotype confirmed by expression of typical endothelial markers such as CD31, vWF and VE-cadherin, typical endothelial cell morphology and performs the same functions³⁹. BOECs are widely used because of their various advantages. In particular, they can be isolated by affected individuals and culture, thus having 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 improves our understanding of vascular disease and facilitates the development of more effective therapeutic strategies⁴⁰. In addition to the production and maintenance of physiological levels of FVIII, ECs play a key role in regulating vascular permeability, forming new blood vessels (angiogenesis) and maintaining extracellular matrix integrity (ECM). New studies reveal that FVIII may play an extracoagulative role, particularly related to the biology of endothelial cells. These studies show that HA patients have reduced endothelial function, including reduced flow-mediated dilation and integral hyperemic velocity (VTI), indicating a reduced endothelial reactivity compared to healthy individuals⁴¹. Additionally, plasma markers also show altered ECM remodeling and vascular integrity, further supporting the hypothesis of systemic endothelial dysfunction in HA³⁸. In vivo investigations on FVIII deficient mice models revealed abnormal vascular remodeling, particularly in the joints. In addition to this, a greater synovial vascular permeability was found during hemarthrosis⁴². This suggests that FVIII deficiency may be associated with a non-physiological angiogenic response, and may therefore contribute to bleeding phenomena and lead to progressive joint damage and inflammation. The use of patient-derived HA-BOECs (HA-BOECs) in recent studies has further investigated the role of FVIII in endothelial function. BOECs taken from patients with severe form HA showed altered behaviour, including reduced tube formation and altered adhesion, which were however restored after exogenous treatment FVIII. In the murine HA models, it was observed that supplementation with FVIII was essential for physiological angiogenesis and maintenance of vascular homeostasis.

In this context, microvascular endothelial cells of the brain (BMECs), a specialized subset of the ECs that make up the blood-brain barrier (BBB) are particularly interesting. The greatest risk for subjects with a severe form of hemophilia is that of intracranial hemorrhages, complication may be caused not only by systemic FVIII deficiency, but also by endothelial dysfunction of intracranial vessels ⁴.

Intracranial Hemorrhages (ICHs)

Intracranial haemorrhages (ICH) are one of the most dangerous consequences of the severe form of hemophilia A and can be either spontaneous or following trauma. They affect about 3-5% of infants with severe HA and are a major cause of morbidity and mortality. ICH can also occur in adult patients with HA, especially patients who have risk factors such as cardiovascular disease or hypertension, possibly leading to disability and in severe cases even mortality, with an approximate mortality rate of 20% ⁴³⁻⁴⁷. It has been estimated that spontaneous intracranial microhemorrhages are more common in patients with HA than in the general population ⁴⁸. Furthermore, although the mechanisms leading to ICH occurrence have not been clarified, there appears to be a close correlation between ICH frequency, degree of FVIII deficiency and patient age ^{49,50}. Since ICH are events that can result from trauma but also occur spontaneously, prophylaxis remains the most effective strategy to prevent their occurrence, especially in children ^{51,52}. Indeed, it has been observed that patients receiving consistent prophylaxis have a lower incidence of ICH than those using non-frequent or no prophylaxis ⁵⁰.

Brain microvascular endothelial cells (BMECs)

BMECs are specialized endothelial cells that constitute the inner lining of cerebral microvessels. These cells possess unique properties compared to peripheral endothelial cells, including high expression of tight junction proteins such as claudin-5 and occludin, low rates of transcytosis, and specific transporter systems ⁵³. BMECs are not only passive structural elements, but key regulators element for BBB function. They respond to environmental stimuli, secrete signaling molecules, and interact dynamically with astrocytes and pericytes. Alterations in BMEC proliferation, morphology, and junctional integrity can significantly impact BBB permeability and vascular stability ⁵⁴. Understanding how FVIII deficiency might affect BMEC behavior offers a mechanistic insight into cerebrovascular alterations observed in HA models. Recent advances in *in vitro* BMEC culture and functional assays provide powerful tools to dissect these effects at the cellular level.

Aim of the project

Hemophilia A (HA) is a monogenic bleeding disorder caused by reduced or absent activity of coagulation factor VIII (FVIII). The severity of HA is directly correlated with the level of residual FVIII activity, with the most severe forms often associated with frequent spontaneous bleeding episodes, including ICHs. Furthermore, recent studies have highlighted endothelial dysfunction in HA patients compared to healthy individuals, suggesting alterations in both macrovascular and microvascular endothelial functions. Although the role that FVIII plays in the coagulative cascade is well known, While the coagulant role of FVIII is well-characterized, its extracoagulative functions remains largely unknown, particularly in maintaining vascular integrity and regulating angiogenesis, remain poorly understood. This knowledge gap becomes relevant when considering the specialized vasculature of the blood-brain barrier (BBB), where endothelial integrity is critical. The primary objective of this thesis is to investigate the role of FVIII in regulating vessel stability and endothelial cell function in the brain's microcirculation. Using in vivo and in vitro models, this project aims to uncover structural and functional differences in the cerebral vasculature between HA and WT mouse models, providing new insights into the vascular dimensions of HA.

Materials and Methods

Animal models

The mouse C57Bl/6 (from Charles River Spa, Calco, Lecco) was used as animal model. Hemophilic A (HA) mice with the same genetic background as wild type mice (WT) were engineered by adding a neomycin resistance cassette after hexone 16 of the eighth factor gene (Bi et al., 1995). All animals were kept under specific pathogen-free (SPF) conditions and all the procedures were reviewed and approved by the Animal Care and Use Committee of Università del Piemonte Orientale (Italian Health Ministry Authorization n.1169/2024-PR, protocol DB064.95).

Preparation of samples for the immunofluorescence staining

To assess structural differences in brain vasculature, brain tissues were collected from both WT and HA c57bl6 mice at various time points: 3, 8, 24, and 52 weeks. After extracting the brains from the cranial box, they were washed in PBS and fixed in paraformaldehyde (PFA) 4% for 2 hours at 4°C . They were then placed in 15% sucrose (prepared in PBS) for 24 hours at 4°C to dehydrate the tissue. Subsequently, they were put in 30% sucrose for another 48 hours , always at 4°C . At this point the brains were included in OCT (inclusion matrix) and stored at -80°C.

Evaluation of brain vasculature

The collected tissues preserved in OCT were sectioned at multiple depths using a cryostat. Immunofluorescence staining was performed using the following antibodies: CD31 (specific for endothelial cells; 1:200) and DAPI for nuclear staining. Tissue sections were cut at a thickness of 15 µm. The immunofluorescences were acquired using a Zeiss Axioscan 7 LED light fluorescence microscope and then analysed with the Zeiss Blue software. This made it possible to observe and analyse the whole section of the sample, and also to select the regions of interest through different magnifications: 20x and 40x.

For each immunofluorescence assay, structural analysis was conducted using ImageJ (<https://imagej.nih.gov/ij/plugins/index.html>). Vessel density was analyzed using the Vessels Analysis plugin which allows to calculate:

- Vessel density: $\text{vessel area} / \text{total area} * 100$
- Vessel length density: $\text{vessel length} / \text{total area} * 100$

Briefly to use this plugin it was necessary to transform the images in black and white. The software is able to define the threshold parameters and turn into blank everything that the microscope has identified as a positive signal. At this point all images are binary and can be processed by the plugin.

Blood brain barrier integrity

To assess the integrity of the Blood Brain Barrier (BBB) on a physiological level, mice in both HA and WT were retroorbited with two physiological solutions containing Dextran molecules of two distinct molecular weights and colors. The first solution contained 4 kDa-FITC Dextran and 150 kDa-TRITC Dextran, and the second solution contained 40 kDa-FITC Dextran and 150 kDa-TRITC Dextran. To induce damage and study vessels response, mice were first treated with 1% DMSO, followed by injections of 4 kDa-FITC Dextran and 150 kDa-TRITC Dextran. Induction with Dextran molecules was performed at three time points (5, 15 and 30 minutes) after administration of DMSO. After 5 minutes of injection of Dextran, the brains were collected, dissected using a cryostat and analysed with ImageJ.

Brain microvascular endothelial cells (BMECs) isolation and characterization

Brain tissues were collected from WT and HA 8 weeks mice. The tissues were mechanically disaggregated in DMEM using a pestle, subsequently were resuspended in DMEM containing collagenase and Dnase for the first enzymatic digestion, for 1 hour at 37°C. Then the cells were enzymatic digested a second time with Dnase and dispase for 1 hour and 30 minutes. BMECs were finally isolated with Percoll gradient, after the cells were collected and plated based on the isolated number of cells, on flasks or plates coated with Collagen type I 0.05 mg/mL. To characterize the collected cells, a portion was stained for CD31 for FACS analysis, from both WT and HA isolated cells. The cells were cultured until passage 3, with the medium being refreshed every two days. After each passage, a subset of the cells was harvested for FACS analysis to evaluate CD31 expression. A first analysis was performed on BMECs, the HA and WT cells were plated respectively in half of a p48well, and the images were taken every 4 hours for 48 hours to follow the growth.

Proliferation

BMECs were cultured at 37°C at pass 0 (p0) to assess their proliferative capacity. The cells were maintained in the Incucyte® for 48 hours and every 4 hours a photo was taken. The rate of confluence was used as an indicator of proliferation. Growth curves were generated and analyzed to compare proliferation rates between WT and HA BMECs. During cell culture, if endothelial identity has been maintained. The expression of CD31 was monitored by flow cytometry (FACS) through each pass. The percentage of CD31+ cells was quantified and compared between the two different groups.

Wound healing

To assess the migration capacity of BMECs, a wound healing test was used. Cells were cultured at 20,000 cells/cm² in a p6 well plate. Once the cells formed a monolayer, they were made mechanically scratched using a p20 pipette tip to create an even area of the wound. The cells were maintained in the Incucyte® for 48 hours and every 4 hours a photo was taken. Moreover, to evaluate the effects of FVIII on wound healing, cells were treated with recombinant FVIII (Nuwiq, Octapharma, 1 U/mL) immediately after the scratch. Four different groups were used for this essay: WT-BMECs untreated, HA-BMECs untreated, WT-BMECs treated with Nuwiq and HA-BMECs treated with Nuwiq. The wound closure area was quantified after 24h using ImageJ software. Results were expressed as a percentage of wound closure compared to the initial wound area.

Statistical analysis

Statistical analysis was performed using the student t test on GraphPad Prism version 9.5.0 (525) for Mac, GraphPad Software (GraphPad Prism , San Diego, CA, USA). The values in the graphs represent the average of one technical triplicate for each mouse, for each time point. The differences were considered significant when p values were $p < 0.01$ (**), $p < 0.001$ (***). When the p-value values were greater than 0.05, the differences were considered not significant (ns).

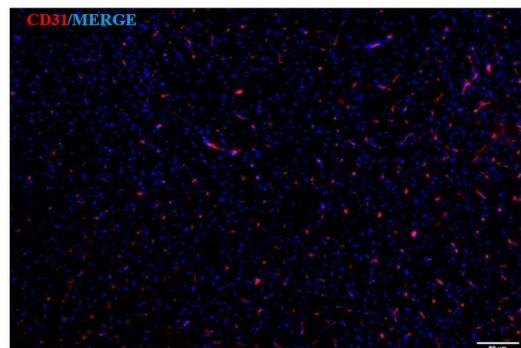
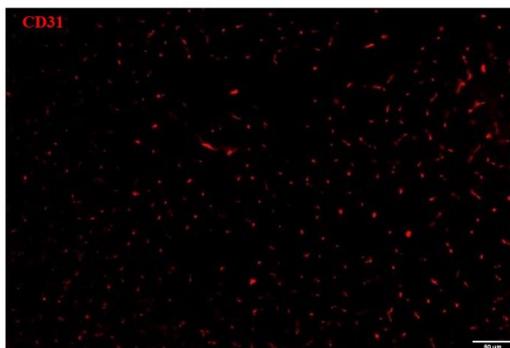
Results

Previous data

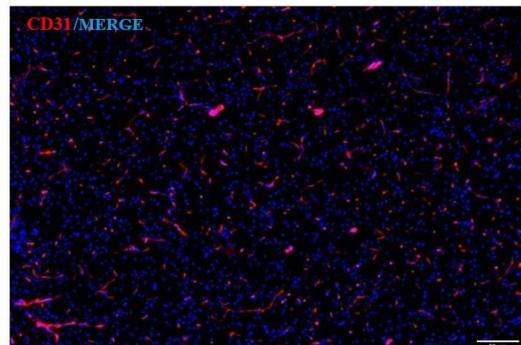
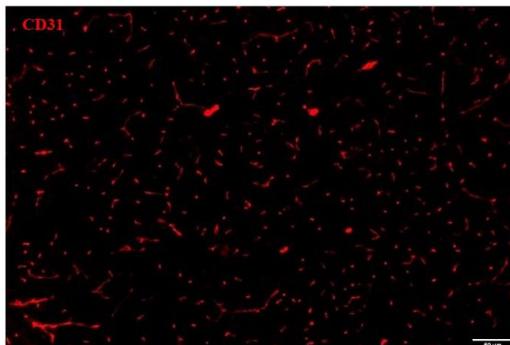
This section will present data from previous studies, which examined differences in cerebral microvascular circulation at 3, 8 and 24 weeks. It has been observed that 8-week haemophilia A (HA) C57BL/6 mice brains show a drastic decrease in the vascular structures and total surface area of the brain microcirculation compared to wild-type (WT) mice. To confirm this difference, HA and WT brain blood vessels were evaluated with immunofluorescence staining for CD31, an endothelial cell marker (Figure 1A). Vascular density and vessel length density were measured using ImageJ, a software or imaging analysis. Figure 1B and figure 1C shown a statistically significantly difference between brain microcirculation of HA and WT mice, moreover, Figure 1B evidence a lower vessel density in HA brain vasculature compared to WT, in mice at 3, 8 and 24 weeks. Figure 1C shows the differences in vessel length density, suggesting that HA brain vessels are shorter than in WT.

1A

HA - 8 weeks



WT - 8 weeks



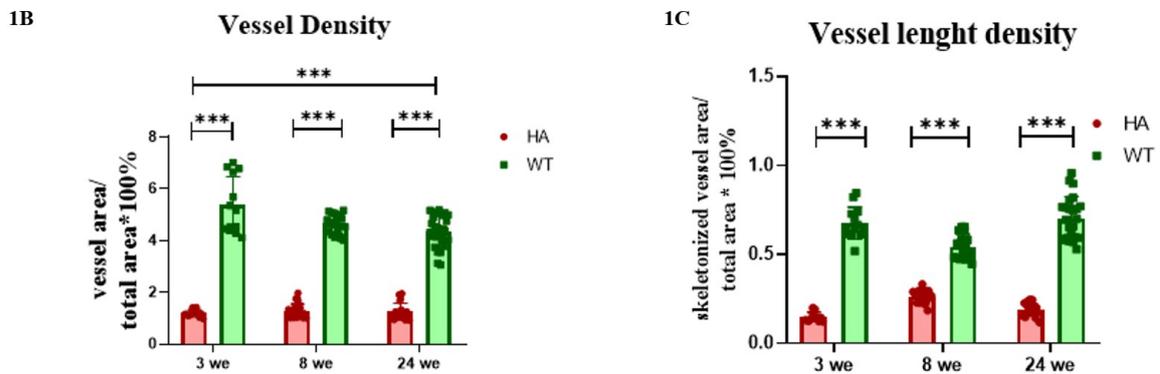


Figure 1. Immunofluorescences that highlight the brain microcirculation in WT and HA mice. (A) Section of mouse microcirculation HA (upper figure) and WT (lower figure) 8-weeks. CD31 (in red) highlights vessels network, DAPI (in blue) highlights the nuclei. (B) (C) Vessel density and vessel length density analysis results obtained using ImageJ software. A significant difference ($P \leq 0.001$) is observed at all time points in both graphs. In vessel density graph (B), it is noted that while WT mice (green) display greater variability across time points, the values for HA mice remain stable over time. The bar represents the \pm s.d. (error bars) of 9 separate experiments.

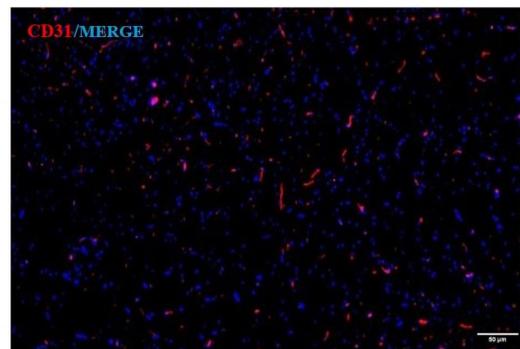
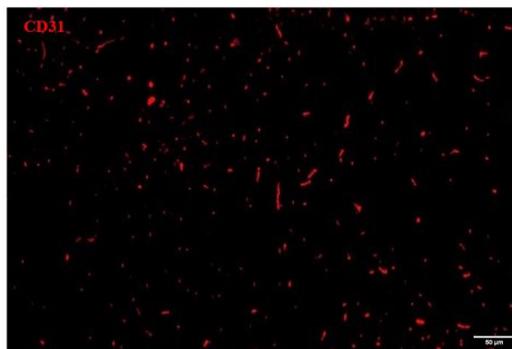
Vessel density 52-weeks mice brain

Brains were collected from 52-weeks HA and WT mice to provide a comprehensive overview of vascular changes throughout the first year of life. This time point was selected based on previous results, allowing for a broader assessment of age-related vascular alterations in these models. Six brains were collected from either HA and WT mouse genotypes at 52 weeks. Immunostaining was performed using anti-CD31 and DAPI. Figure 2A shows representative images of brain microcirculation in 52 weeks HA (upper figure 2A) and WT mice (figure 2A below). It is possible to observe a difference in vessel density and length. HA brain vessels appear shorter and in a lower number compared to WT which seems to be also more ramified. To ensure a comprehensive evaluation of brain vasculature, each brain was sectioned at a consistent thickness of 15 μm across three distinct depths and from each section images were taken in three specific brain regions. ImageJ software was used to analyse the images. The "vessel analysis" plugin has been utilized to quantify the brain vasculature, by measure the vessel density and the vessel length density. Vessel density (vessel area/tot area*100) has been evaluated to determine the number of blood vessels, revealing patterns in vascular distribution

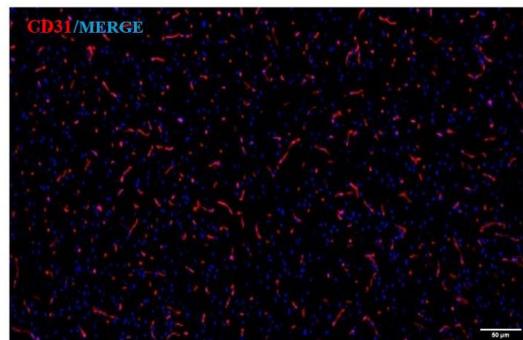
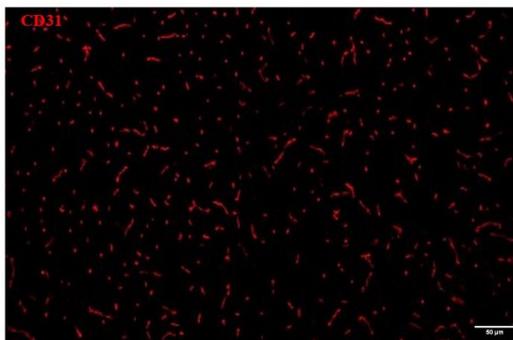
within the analysed tissue while Vessel length density (skeletonized vessel area/total area*100) has been calculated to evaluate the total length of all detected vessels, offering insight into the complexity and extent of the vascular network. This staining highlighted differences between HA and WT mice and, as illustrated in graphs 2B, HA brains exhibit a significantly reduced number of blood vessels compared to WT brains. Figure 2B shows the value of the vessel length density in 52 weeks HA and WT mice, the graph exhibits a significantly difference between vessel length of the two groups, in details HA length vessel is lower compared to WT. To have a general overview of the brain vasculature changes and eventually differences between HA and WT mice, all the time points obtained were unified in a single graph (Figure 2D and 2E). In figure 2D is possible to observe that, while in HA brain the vessel density remains constant throughout the year of life, WT brain vessels show a significantly difference in number at different time points, suggesting a dynamic trend in WT vasculature structure which seems to be lost in HA brain microcirculation.

2A

HA - 52 weeks



WT - 52 weeks



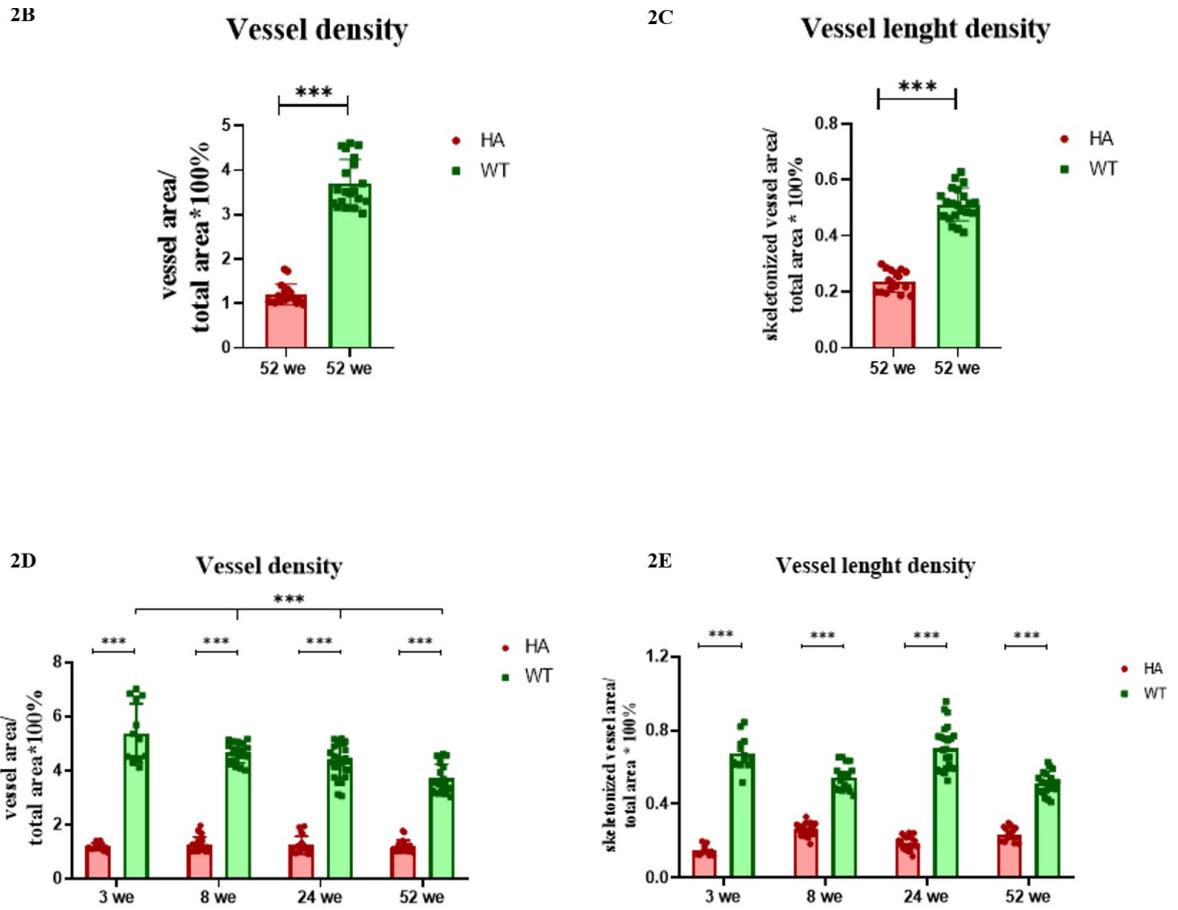
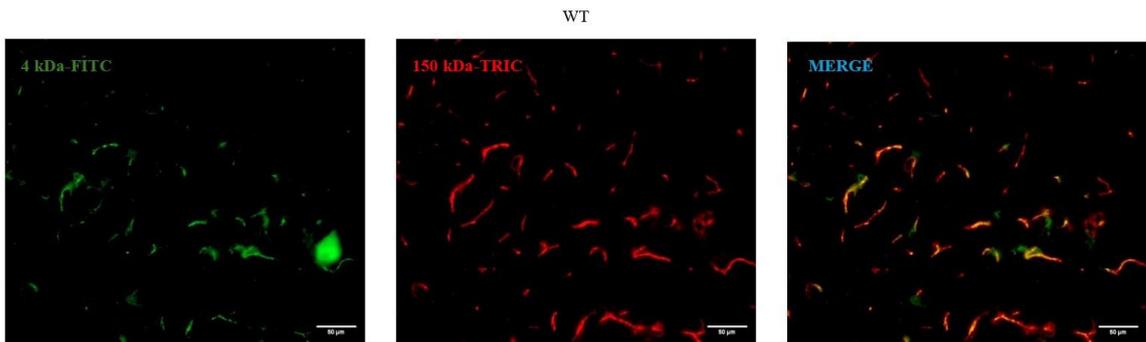
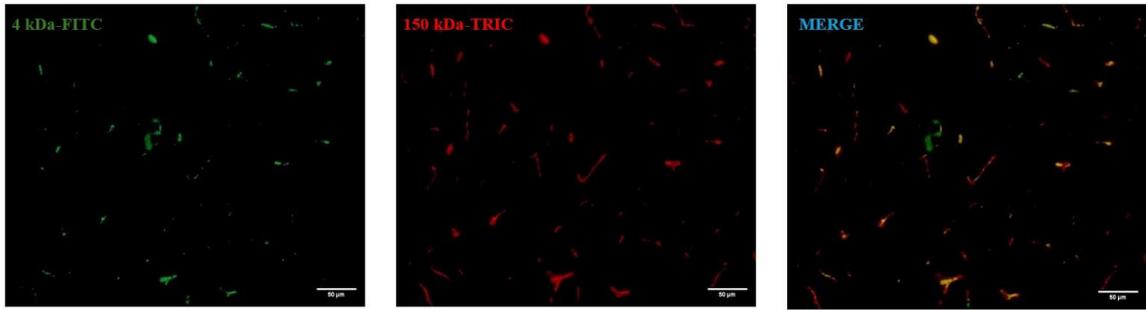


Figure 2. Immunofluorescences that highlight the brain microcirculation in WT and HA mice. (A) Section of mouse microcirculation HA (upper figure) and WT (lower figure) 52-weeks. CD31 (in red) highlights vessels network, DAPI (in blue) highlights the nuclei. (B) (C) Vessel density and vessel length density analysis results obtained using ImageJ software. (D) (E) Vessel density and vessel length density analysis results of the different time points compared in a single graph. A significant difference ($P \leq 0.001$) is observed in both graphs. The bar represents the \pm s.d. (error bars) of 3 separate experiments.

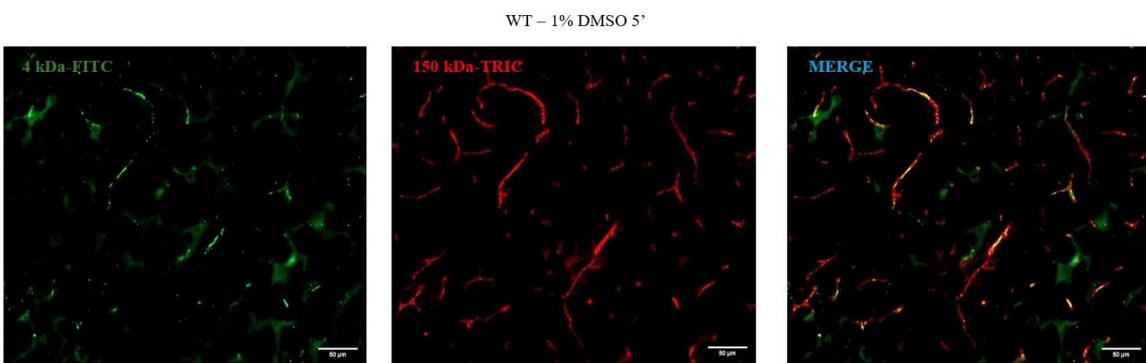
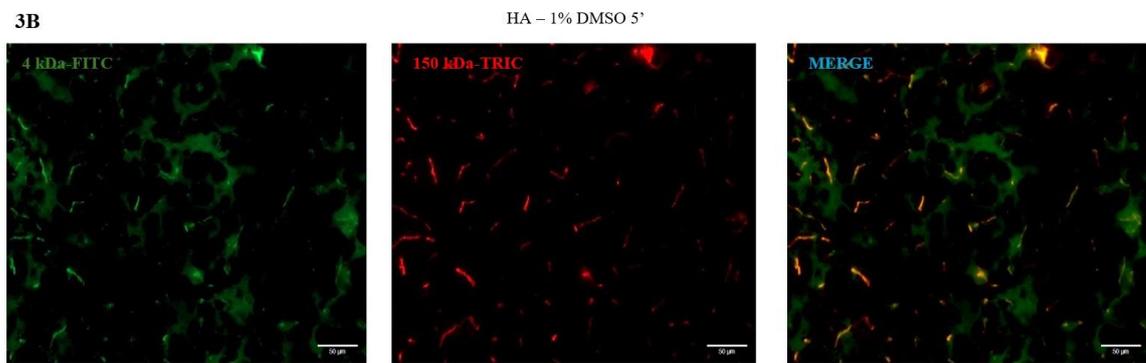
Blood Brain Barrier Integrity

Given the observed differences in vascular density between HA and WT mice, assessing blood-brain barrier (BBB) permeability was essential to determine whether these structural variations observed above could affect the BBB functionality. To assess BBB integrity under both physiological and damage-induced stress, fluorescence dextrans of two different molecular sizes were used. This approach allowed for a comparative evaluation of BBB permeability and structural integrity across varying molecular weights. Under physiological conditions (Figure 3A), only a minimal extravasation of 4 kDa-FITC dextran is observed in both HA and WT brain sections, which is not significant between the two strains. In addition, dextran 150 kDa-TRITC remains confined inside the vessels, not showing extravasation thus demonstrating that the integrity and selective properties of the barrier remain intact for both HA and WT brains. Figure 3B shows what happens after an injury induced by 1% of DMSO. The degree of extravasation of 4 kDa-FITC in this case increases considerably in the brain of the HA mice, with clear diffusion within the tissue, present already 5 minutes after injection. In comparison, the extravasation of 4 kDa-FITC in WT brains shows a moderate increase in permeability, showing a higher degree of barrier integrity. It is interesting to note that the level of extravasation for WT brains, both on a physiological level and after damage, varies slightly during different time points, compared to HA brains. In figure 3C is possible to observe that HA mice brain vessels leakage progressively increased over time, with significantly higher levels of 4kDa-FITC extravasation observed at 5, 15, and 30 minutes after injury compared to WT controls ($***P < 0.001$). As can be seen from Figures 3A and 3B, the 150 kDa-TRITC dextran molecules remain localized within the vessels, without extravasation. This occurs in all time points considered and for both, physiological conditions and after damage. These findings indicate that even mild damage is sufficient to perturb the BBB in HA mice.

3A



3B



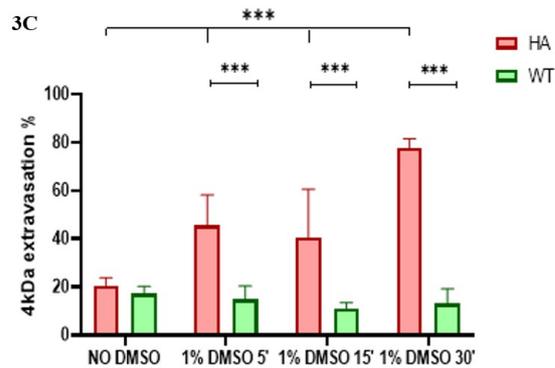


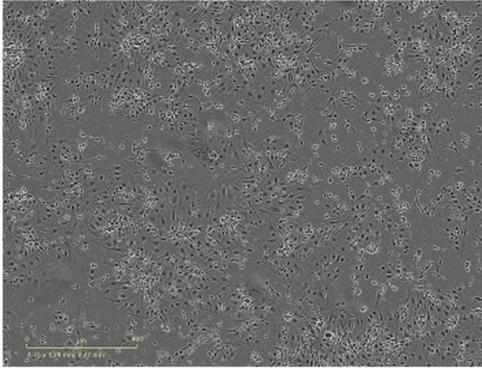
FIGURE 3. Representative images of HA and WT brains treated with 4kDa-FITC and 150kDa-TRITC dextran are shown under physiological conditions. A slight extravasation of 4kDa-FITC can be observed (Figure .2.a). In a damaged condition (1% DMSO), a marked extravasation is noticeable in HA brains compared to WT brains (Figure 2.b). The level of extravasation was assessed at 5-, 15-, and 30-minutes post-injury (1% DMSO) (Figure 2.b). The graph shows that extravasation significantly increases over time in HA mice compared to WT mice $P \leq 0.001$. The bar represents the \pm s.d. (error bars) of 3 separate experiments.

Brain Microvascular Endothelial Cells (BMECs) isolation and characterization

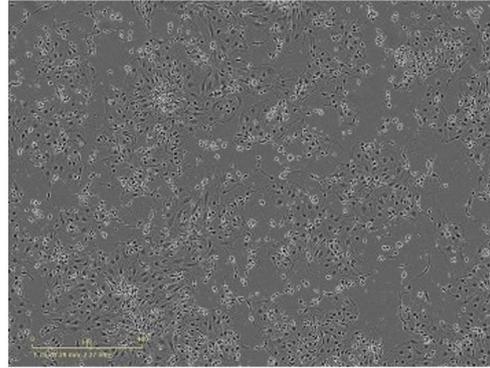
Once the structural and functional differences between HA and WT were established, the next step was to investigate the cells that made up the brain microcirculation. Figure 4A shows two representative images of BMECS at passage 0 (p0) and at passage 3 (p3), it is possible to observe a higher density of WT-BMECs compared to HA-BMECs and it is evident in both a morphology changes through the passages. At the microscope observation at p0, the cells were smaller, more compact and crowded (figure 4A). However, as they progressed through passages, their morphology changed drastically both HA and WT BMECs, became larger and more widespread, covering the entire surface of the plate. To confirm their endothelial nature, at each passages the cells were characterized at FACS through the CD31 marker. Another way for BMECs characterizations is immunofluorescence staining that show that cultured cells are >98% CD31⁺/CLDN5⁺ and no CD45⁺ cells persist in the plate (Figure 4B). Only a small percentage of pericytes (alphaSMA⁺) simultaneously grow with BMECs (Figure 4B). The percentage of cells expressing CD31 increases at each pass as shown in the bar graph in figure 4D, demonstrating a progressive increase of BMECs culture purification.

4A

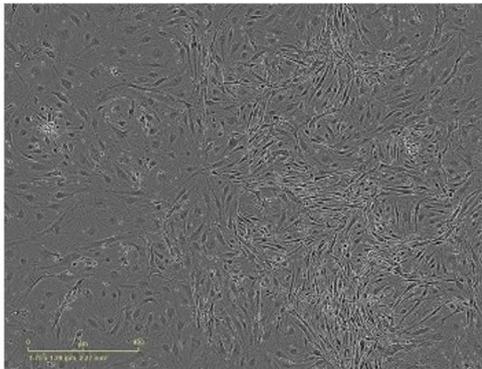
WT BMECs – p0



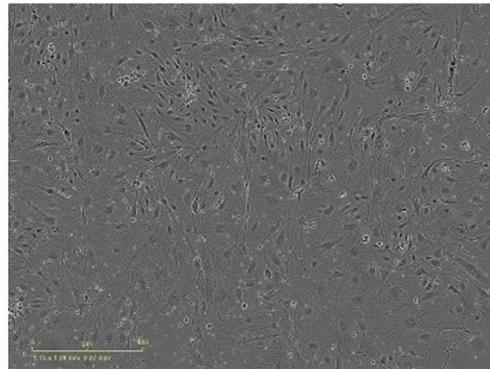
HA BMECs – p0



WT BMECs – p3



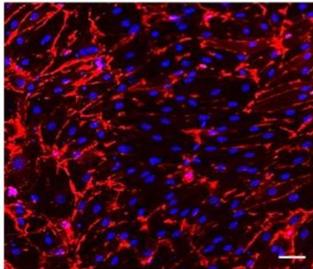
HA BMECs – p3



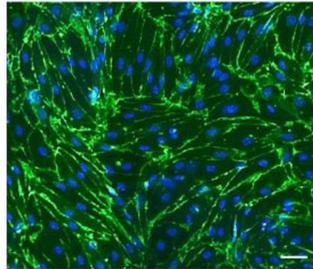
4B

WT BMECs

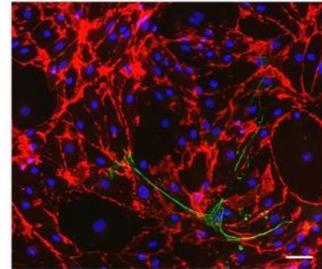
CD31/CD45/DAPI



CLDN5/DAPI

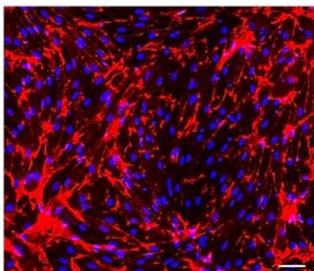


CD31/alphaSMA/DAPI

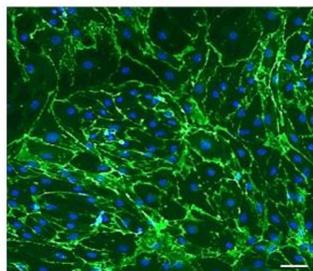


HA BMECs

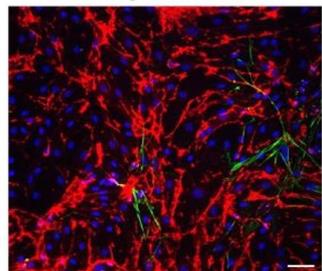
CD31/CD45/DAPI



CLDN5/DAPI



CD31/alphaSMA/DAPI



Functional assays

The proliferative capacity of BMECs was assessed through real-time growth monitoring. This monitoring was performed on p0 for 48 hours, through the use of the Incucyte instrument. Figure 4C shows that HA-BMECs have a significantly lower rate of proliferation than WT-BMECs ($P \leq 0.001$). WT-BMECs show a growth rate almost twice that of HA-BMECs, indicating a substantial reduction in proliferation capacity in the HA group. To evaluate the maintenance of endothelial identity through in vitro expansion, CD31 expression was analysed by flow cytometry from passage 0 to 3. Figure 4D shows that both HA and WT BMECs have a comparable expression of CD31 at p0 and p1, while for p2 and p3, WT BMECs showed an increase in CD31+ cells compared to HA BMECs ($P \leq 0.001$).

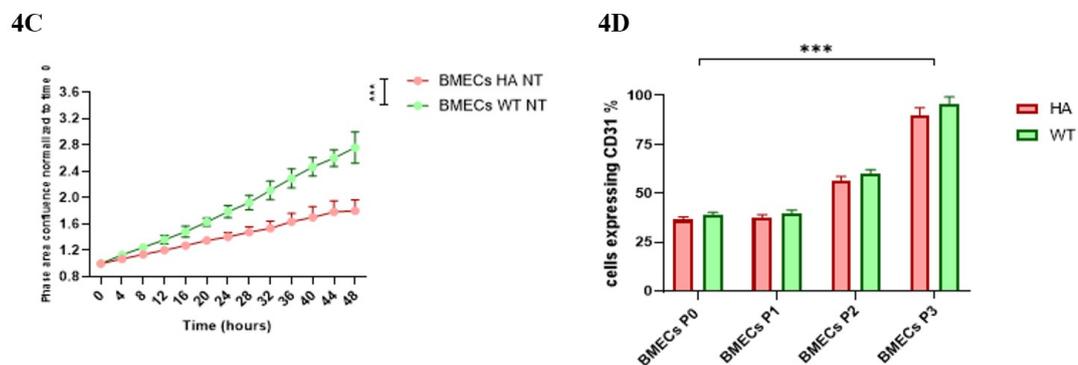


FIGURE 4. The figure shows representative images of BMECs isolated from HA and WT mice, cultured up to passage 3 (4A). Immunofluorescence for CD31, CLDN5, CD45 and alphaSMA on BMECs obtained from WT and HA mice after 4 days of culture. Scale bar= 200 μ m (4B). The graphs display the results of a proliferation assay performed on BMECs at passage 0, indicating a significant decrease in cell proliferation in HA BMECs compared to WT (4C) ($P \leq 0.001$). BMECs were characterized after each passage, assessing CD31 expression via FACS. The graph illustrates the percentage of CD31-expressing HA and WT BMECs across different passages, with a significant difference observed at p0 and p1 compared to p2 and p3 (4D) ($P \leq 0.001$). The bar represents the \pm s.d. (error bars) of 3 separate experiments.

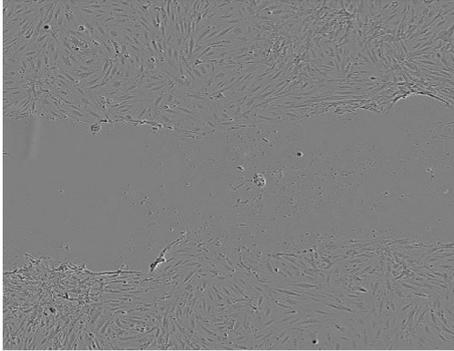
BMECs Wound Healing assay

To assess the migratory capacity of BMECs, a scratch wound healing test was performed on HA and WT-BMEC monolayers. Figure 5A shows the representative images of the wound healing assay obtained at time 0 and showing the extent of wound closure after 24 hours. HA-BMECs figures show a decreased level of wound closure indicating a

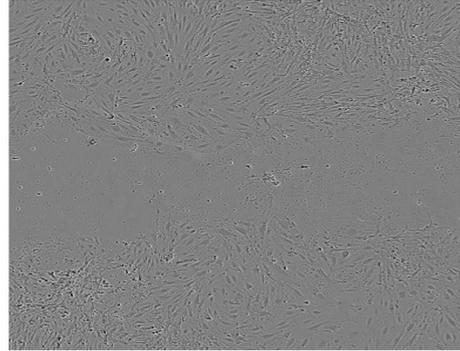
limited migratory activity of these cells, by contrast, WT-BMECs showed greater wound closure. Recombinant factor VIII (Nuwiq, 1 U/mL) was tested for both WT and HA-BMECs (figure 5B). The addition of Nuwiq greatly improved the wound healing in both HA and WT cells. HA-BMECs treated with Nuwiq showed a visible increase in wound closure within 24 hours, suggesting an increased motility. WT-BMECs treated with Nuwiq exhibited almost complete closure of the scratch area, further increasing their already higher baseline migration capacity. The quantification of wound closure after 24 hours is shown in the bar graph shown in Figure 5C. For untreated BMECs, HA-BMECs had only about 25% closure, significantly less than the 38% observed in untreated WT-BMECs. After Nuwiq treatment, the HA-BMECs increased wound closure by about 67%, similar to the WT-BMECs which reached over 70%. Statistical analysis confirmed that these differences are highly significant between the untreated and the treated groups ($P \leq 0.001$), thus demonstrating that Factor VIII supplementation significantly ameliorate the HA-BMECs migratory deficit , also improving WT-BMECs migration.

5A

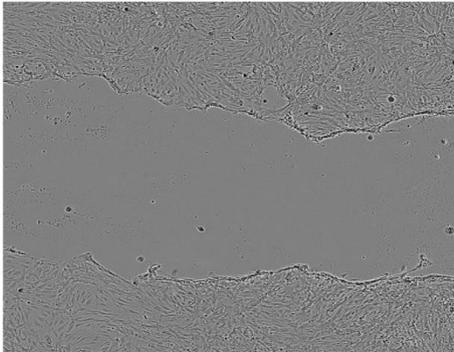
BMEC HA - 0 H



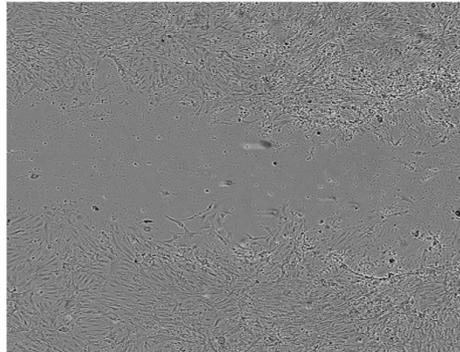
BMEC HA - 24 H



BMEC WT - 0 H

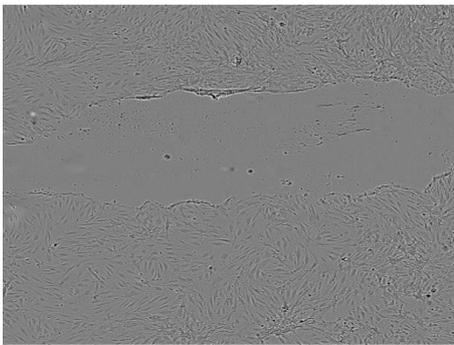


BMEC WT - 24 H

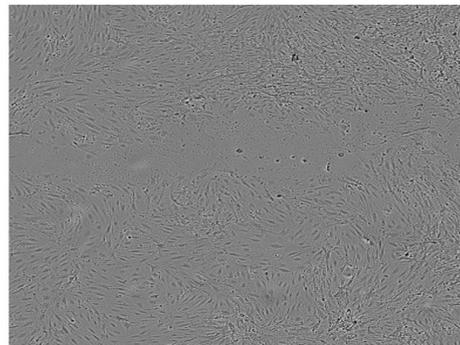


5B

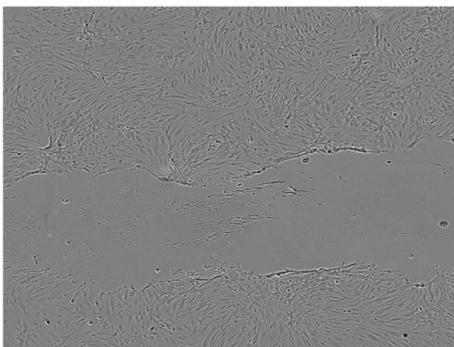
BMEC HA + Nuwiq 1U/mL - 0 H



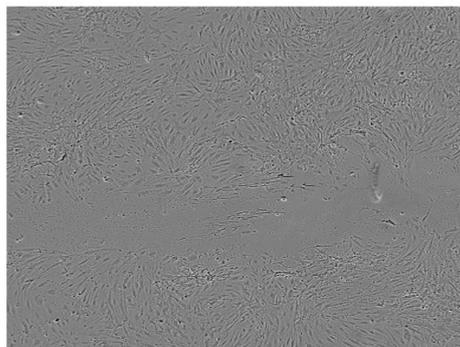
BMEC HA + Nuwiq 1U/mL - 24 H



BMEC WT + Nuwiq 1U/mL - 0 H



BMEC WT + Nuwiq 1U/mL - 24 H



5C

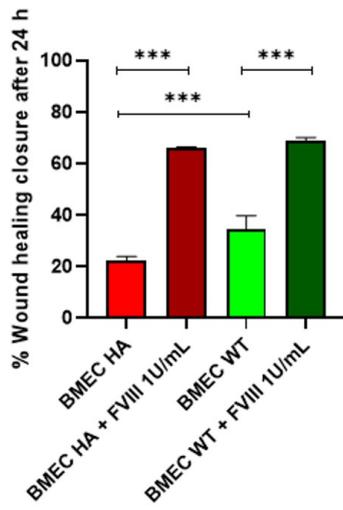


FIGURE 5. The figure shows representative images of wound healing assay performed on HA and WT-BMECs untreated (5A) and treated with Newiq 1 U/mL (5B). The graph (5C) display the results of wound healing closure after 24 hours, indicating a significant decrease in cell migration in untreated HA-BMECs compared to WT ($P \leq 0.001$). The same graph shows the increase in the closure rate of HA-BMEC and WT-BMEC after treatment with Newiq. The bar represents the \pm s.d. (error bars) of 3 separate experiments.

Discussion

Hemophilia A (HA) is a monogenic bleeding disorder caused by the deficiency or totally absence of blood clotting factor VIII (FVIII) ¹. Based on the residues level of FVIII, the severity of the disorder can be classified in three forms: a mild HA, which occurs when the residual activity of FVIII varies from 5% to 40% and is generally diagnosed in adulthood, a moderate form of HA, which occurs when the FVIII varies from 1% to 5%, so those affected have moderate bleeding in particular and after trauma of low and medium extent or after surgery and the severe form, where FVIII activity is less than 1% and is often diagnosed at birth or within the first two years of life and is characterized by frequent bleeding, in particular at the level of the skull and patients may present frequent subcutaneous hematomas due to spontaneous bleeding ⁴. As an X-linked condition, it primarily affects males, resulting in frequent and potentially life-threatening bleeding episodes, particularly in joints (hemarthroses) and intracranial spaces. The clinical manifestations of hemophilia patients are mainly spontaneous or trauma-induced bleeding in different parts of the body depending on the severity of the disease ⁸. Particularly important with regard to the severe form of HA are the spontaneous episodes of bleeding that may occur within the skull. These intracranial hemorrhages (ICH) represent a critical condition, especially for children. Estimates reveal that spontaneous intracranial microhemorrhages are more common in patients with HA compared to general population ⁴⁸. Recent clinical and pre-clinical findings have revealed that HA individuals may encounter vascular issues. In HA, there is growing evidence that ECs, in addition to their coagulative role, play a key role in abnormal vascular responses. This includes increased permeability and compromised repair mechanisms, which can contribute to the severity of bleeding episodes and hinder recovery after vascular lesions ^{3355 34}. The results obtained from these studies indicate significant alterations in both macrovascular and microvascular endothelial functions. Nowadays, the role that FVIII plays in the homeostasis and functionality of ECs remains largely unexplored. While some research has investigated the extra-coagulative effects of FVIII in bone remodelling and macrophage polarization, more studies are required to fully comprehend the implications of FVIII in endothelial function ⁵⁶. Endothelial play a crucial role in the central nervous system, where endothelial cells are specialized to form the blood–brain barrier (BBB) ²⁸, a crucial component in maintaining cerebral homeostasis. The endothelial cells, in this system at capillary level, are distributed to form a continuous endothelium, with the

function of preventing the entry of potentially harmful elements present in the blood and allowing the passage of necessary substances to the brain, thanks to their specialized junctional complexes (tight junctions, and gap junctions) ²⁹. Based on this, to better understand this dysfunction of the endothelium and ICHs phenomenon, we investigated the physiological differences between the healthy and hemophilic cerebral vasculature in C57BL/6 mice. Our previous results showed that there was a substantial difference in vascular length and vascular length density, using brains of wild type (WT) and HA mice of 3, 8 and 24 weeks old. Especially, by examining histological sections of the two types of brain and comparing vascular structures, highlighted by specific markers such as CD31, it was possible to appreciate a drastic decrease of the vascular structures and total surface area of the cerebral microcirculation HA compared to WT. To have a complete overview of the first year of life, these same histological analyses were achieved to confirm this result in 52 weeks of mice. 52 weeks HA mice -continue to show reduced number of blood vessels and these vessels are shorter in length than those in WT mice. Importantly, what we appreciated by putting all these results together, from 3 weeks up to 52 weeks is that, while in HA brain the vessel density remains constant throughout the life, WT brain vessels show a significant difference in number at different time points, suggesting a dynamic trend in WT vasculature structure which seems to be lost in HA brain microcirculation. The structural assessment was the initial step, and in order to investigate cerebral vascularization further, we assessed the integrity of the blood-brain barrier, to determine whether the structural differences observed above could affect the functionality of the BBB. Under normal physiological conditions, the BBB appears intact in both animal models, with no extravasation observed, thus demonstrating that the integrity and selective properties of the barrier remain intact for both HA and WT brains. The differences were observed after both groups of mice were subjected to a mild injury. It has been discovered a notably higher level of permeability in HA mice compared to WT mice. Although the BBB initially appears comparable between the two groups, this finding suggests that even minor injury can significantly compromise the barrier's integrity in HA mice, leading to higher susceptibility to damage while in WT brains shows a moderate increase in permeability, suggesting a higher degree of barrier integrity. Furthermore, the observed increase in extravasation time of 4kDa-FITC dextran in HA mice highlights the possibility that endothelial cells in these mice showed a slower or less effective response to injury repair, compared to the WT counterpart, which in both physiological level and after damage, varies slightly during different time points. Once the structural and

functional differences between HA and WT were established to better understand the mechanisms underlying this increased permeability and impaired recovery in HA mice, it was essential to focus on cells responsible for maintaining the blood-brain barrier's integrity, the brain microvascular endothelial cells (BMECs). BMECs are specialized subset of the ECs that make up the blood-brain barrier and that play a central role in preserving the tightness of the BBB. Their isolation allowed us to investigate how endothelial dysfunction may contribute to brain vascular complications in hemophilia A. Thanks to the improvement in our laboratory of the protocol for their isolation, we were able to isolate the cells correctly and kept them in culture until passage 3. In culture it was possible to observe a higher density of WT-BMECs compared to HA-BMECs. However, as they progressed through the next steps, their morphology changed drastically both HA and WT, became larger and more widespread, covering the entire surface of the plate. The proliferation assay demonstrating that HA-BMECs exhibit a lower proliferation rate compared to WT-BMECs, suggesting an intrinsic defect in their growth capacity. Flow cytometry analysis (FACS) confirmed the endothelial identity of cells in culture through expression of CD31. In all steps, the expression of CD31 was detected by confirming an increased expression or membrane localization at each passage. This indicates a progressive purification of the BMEC population over time. These results demonstrate the successful isolation, expansion, and characterization of BMECs from both WT and HA mice. Despite the lower initial density of HA-BMECs, which may reflect intrinsic functional deficits, the progressive increase of CD31+ marker across the various passages, provides a critical starting point for utilizing these cells in understanding the pathophysiology of vascular dysfunction through functional tests such as permeability, migration and tube formation. To further study functional alterations in BMECs derived from HA mice, functional assays such as proliferation and migration were performed, which are key processes involved in vascular development and repair. Following the real-time proliferation of BMEC, it has been observed that HA-BMECs proliferate at a significantly lower rate than their WT counterparts, which have exhibited almost twice the growth rate of HA-BMECs. This altered proliferation suggests one of the possible causes that would impair vascular remodelling and regeneration, particularly under stress or injury conditions. During proliferation, to assess whether the endothelial phenotype was maintained during in vitro expansion, we analysed CD31 expression through passages by FACS. HA that WT BMECs showed a CD31 expression comparable to the first steps (P0 and P1) while a difference was noted at P2 and P3, where WT-BMECs showed a

significant increase in CD31+ expression compared to HA-BMECs. This suggests that HA BMEC has impediments in maintaining the endothelial phenotype, as opposed to WT BMEC which maintains it stably during expansion. Additionally, the wound healing analysis provided further information on the migratory capacity of BMECs. HA-BMECs showed a marked reduction in wound closure compared to WT-BMECs after 24 hours of monitoring, showing a migratory deficit in the HA group. This reduced motility may be associated with the results obtained from barrier integrity in which a slowdown in vascular repair and an increase in BBB permeability were observed as a result of injury. Interestingly, treatment with recombinant factor VIII (Nuwiq) led to a significant increase in wound closure in both HA and WT-BMECs. In HA-BMECs the increase was almost three times, restoring migratory capacity and bringing it to levels comparable to untreated WT cells. Surprisingly, even treated WT-BMECs have shown an improved migration capacity. These results suggest that FVIII deficiency in HA mice not only impaired haemostasis but also impairs endothelial function. In conclusion, these findings reinforce the hypothesis that endothelial dysfunction is a key component of FVIII deficiency and further demonstrate that FVIII supplementation can partially restore proper endothelial behaviour.

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