

Propranolol therapy for cerebral cavernous malformations

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Abstract. Cerebral cavernous malformations (CCMs) are vascular malformations characterized by the abnormal growth of vascular structures in the central nervous system. However, the precise mechanism(s) responsible for the development of CCM vascular abnormalities remain poorly understood. Although the mechanisms of action of propranolol in CCM have not yet been fully explored it is not commonly prescribed, it has been shown to be effective in children and appears to play a protective role in the prevention of CCM-derived hemorrhage in adults. The present study performed *in vitro* and *ex vivo* assays in order to examine the effects of propranolol on endothelial cells (ECs). The percentage of CD14⁺/CD31⁺ cells and the levels of VEGF in the peripheral blood (PB) of a child patient with CCM, with recurrent seizures and hemorrhages, who was maintained under propranolol therapy, were also analyzed. In addition to the effects of propranolol on differentiated ECs, and the decrease angiogenic-related features *in vitro* and *ex vivo*, it was observed that in the PB of this patient, propranolol administration decreased the percentage of circulating cells sharing monocytic and EC features (CD14⁺/CD31⁺ cells), as well as the VEGF levels; this was concomitant with a good prognosis and with the reversion of CCM lesions. A decrease in VEGF levels by propranolol may also be involved in the impairment of the recruitment of CD14⁺/CD31⁺ monocytes functioning as endothelial progenitor cells to sustain the

vascular lesion. On the whole, the present study demonstrates that propranolol impairs angiogenesis *in vitro* and may thus be a useful tool for the clinical management of CCM. Moreover, the present study highlights the monitorization of the levels of CD14⁺/CD31⁺ monocytes and VEGF levels as a useful tool for predicting the clinical efficacy of propranolol in patients with CCM.

Introduction

Cerebral cavernous malformations (CCMs) present a relatively low prevalence (0.16-0.5%), accounting for 5-15% of all central nervous system vascular malformations (1-3). The disease is characterized as low-flow vascular malformations composed of blood-filled sinusoidal locules known as 'caverns'. At the histological level, CCM is characterized by the lack of mural elements of mature vascular structures (3).

The major clinical presentations are epilepsy, headaches or focal neurological deficits; however 30% of the patients are asymptomatic or presents non-specific headache (4). During disease progression, the growth of vascular malformations is associated with recurrent hemorrhages (annual hemorrhage rate of 0.6-11%/patient/year) (3,5), which is considered to be a consequence of the immature vascular network constituting the CCM lesions (6). In the management of this pathology, it is also important to take into account changes in arterial pressure, which can alter the hemorrhage propensity and patterns (7). Depending on the anatomical localization of the CCM, patient management relies on surgical resection, observation and symptomatic treatment. In surgical inaccessible lesions, drugs such as statins, anti-angiogenic agents or vitamin D3 have been tested, although none have revealed clear benefits (8-11).

The beneficial use of propranolol in childhood hemangioma, a close pathological counterpart of cavernous malformations, supports the putative use of propranolol in the management of patients with symptomatic CCM (12). Propranolol administration in patients with CCM, although not commonly prescribed, has effectively been used in children and appears to play a protective role in the prevention of CCM-derived hemorrhaging in adults (13-18).

A common feature of propranolol-sensitive vascular tumors, such as hemangioma and CCM, is the distinctive

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expression of CD15-positive ‘vasculogenic zones’ (19-21). Of note, the *in vitro* gain of CD15 is followed by embryonic stem cell differentiation into endothelial cells (ECs) (22), indicating the putative involvement of endothelial progenitor cells (EPCs) in the development of vascular malformations. The association between CD15-positive cells and neo-vessels formation is not novel; it was described, >40 years ago in immature vessels of the placenta (23,24); the effects of propranolol on the placental regression have also long been described (25).

The identification of EPC subsets in peripheral blood (PB) is not yet clear. The authors have previously demonstrated that in tumors and normal tissues, some ECs simultaneously express CD14 (monocytic marker) and CD31 (EC marker), indicating mixed features between monocytes and ECs (26). More recently, it was demonstrated that monocytes can differentiate into ECs and be incorporated into blood vessels (27). These studies indicate the underestimation of monocytes as a relevant source for vascular growth. In fact, due to their 2-10% prevalence in PB (28) and compared to the estimated 0.002% of EPCs proposed by other studies (29-32), monocytes are putatively the most representative EPC subgroup in PB. Moreover, some studies have demonstrated that CD15 is also expressed in monocytes (33,34), with its levels being increased in pathological conditions (35). Notably, in tumors, CD14 immune cells are also CD15-positive, clearly indicating a subset of monocytes/macrophages (36). These observations indicate that CD15 cells in the ‘vasculogenic zone’ are in fact monocytes functioning as EPCs, contributing for blood vessel formation in CCM.

Hemangiomas and CCM share phenotypical characteristics, being both composed of a mixture of abnormal dilated capillary vessels with disorganized ECs and pericytes (37-39). The exact mechanisms that regulate the development of vascular abnormalities remain poorly understood. It is known that during the growth phase of hemangiomas, the increased expression of fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) is associated with ECs and interstitial cell proliferation (37). The effect of propranolol in the reversion of vascular malformations is putatively associated with a decreased expression of FGF and VEGF, impairing EC migration, proliferation and reorganization, which in turns leads to vasoconstriction (involution phase) (12,40-42).

Since the natural evolution of CCM is chronic and unpredictable, the follow-up of patients with CCM involves the long-term clinical and imagiological evaluation with magnetic resonance imaging (MRI) (12). In an attempt to identify a suitable follow-up method, the monitorization of the levels of CD14⁺/CD31⁺ monocytes in the PB of patients with CCM is proposed. Considering that the authors recently published a study demonstrating that monocytes are viable EPCs (27), it was hypothesized that circulating CD14⁺/CD31⁺ monocytes function as EPCs and contribute to the development of CCM lesions.

Materials and methods

PB processing and cell characterization. The PB of a 13-year-old Caucasian girl with CCM was collected and analyzed, between 2013 and 2020, after obtaining informed consent from her parents at the Neuropediatrics Department

at the Portuguese Institute of Oncology of Lisbon, Francisco Gentil (IPOLFG; ethics approval was obtained from the IPOLFG Ethics Committee; UIC-1137); her parents also agreed to the publication of the case study. The PB was centrifuged at 155 x g for 5 min, at room temperature, and serum was then stored at -20°C until further analysis. The cell pellet was resuspended in 45 ml 1X RBC lysis buffer (786-1701, G-Biosciences) and incubated for 15 min in the dark, at room temperature. Subsequently, the resuspended cells were centrifuged at 155 x g for 5 min, at room temperature, washed twice with 1X phosphate-buffered saline (PBS) and incubated with anti-CD14-FITC (1:100; cat. no. 555397, BD Biosciences) and anti-CD31-APC (1:100; cat. no. FAB3567A, R&D Systems, Inc.) antibodies in 0.5% bovine serum albumin (BSA; BSAV-RO; Merck KGaA)-PBS (v/w) at 4°C for 20 min in the dark. Immunolabelling was evaluated using a flow cytometer (FACSCalibur, BD Biosciences) and the data were analyzed using FlowJo X v10.0.7 software (<https://www.flowjo.com/>). PB cells from healthy blood donors (at least two by measurement) were used, under consent, as normal controls. A total of 16 controls, male and female, with an age range between 18 and 40 years, collected between 2013 and 2020; sample collection was performed (four donors at each time point of follow-up) on the same date with the CCM patient blood collection.

EC culture. Human umbilical vein ECs cells (HUVECs; CRL-1730, ATCC) were cultured in endothelial cell growth basal medium-2 (EBM-2: CC-3156, Lonza Group, Ltd.) supplemented with EGM-2 SingleQuots Supplements (CC-4176, Lonza Group, Ltd.), which included 2% fetal bovine serum (FBS- CC4101A, Lonza Group, Ltd.), and maintained at 37°C in a humidified atmosphere with 5% CO₂. The cells were used until passage 10 and were detached with 0.05% Trypsin-EDTA 1X (25300-054, Invitrogen; Thermo Fisher Scientific, Inc.). For the experimental conditions, the cells were cultured in the presence or absence of 100 µM propranolol (P8688; Sigma-Aldrich; Merck KGaA), as previously described (43).

Monocyte isolation and culture. Monocytes were isolated from PB collected after obtaining consent from healthy donors, from 2013 to 2020, at the Immune-Hemotherapy Department at Portuguese Institute of Oncology of Lisbon, Francisco Gentil (IPOLFG). Ethics approval was obtained from the IPOLFG Ethics Committee; UIC-1137). PB mononuclear cells (PBMCs) from blood samples were separated using Histopaque-1077 (10771, Sigma-Aldrich; Merck KGaA), followed by magnetic monocyte isolation using the Monocyte isolation kit II (130-091-153, MACS Technology; MiltenyiBiotec, Inc.), according to the manufacturers' protocols. Monocytes were cultured in EBM-2 (CC-3156, Lonza Group, Ltd.) plus EGM-2 SingleQuots Supplements (CC-4176, Lonza Group, Ltd.) and with 2% FBS (CC4101A, Lonza Group, Ltd.), 50 ng/ml VEGF (V7259, Sigma-Aldrich; Merck KGaA) and 10 U/ml heparin (H3149, Sigma-Aldrich; Merck KGaA). The cells were maintained at 37°C, in a humidified atmosphere with 0.5% CO₂. Hydrogen peroxide (H₂O₂; 15 µM; 1.07210.0250, MerckKGaA) was used as a reactive oxygen species (ROS) generator, as previously described (27). The inhibitory effects of propranolol (100 µM; 16 h) on monocyte differentiation capacity in these particular culture conditions

have been previously published by the authors. The differentiation process was confirmed by measuring the levels of expression of the endothelial marker, vWF, as previously reported (43).

Determination of VEGF levels. The concentration of VEGF in PB serum and in the culture medium conditioned by monocytes, isolated as described above, was evaluated using the Human VEGF Quantikine ELISA kit (DVE00, R&D Systems, Inc.), according to the manufacturer's instructions. PB serum from healthy blood donors (the same donors used to determine blood cell markers), was used as normal controls and for cell supernatants, cells under control conditions were maintained in H₂O₂ and propranolol-free media.

Cell proliferation assay. The determination of cell proliferation was calculated using the ratio of total and Ki67⁺ nuclei. Briefly, HUVECs (5x10⁴ cells/well) were cultured on glass slides coated with 0.2% gelatin and fixed in 2% paraformaldehyde for 15 min at 4°C, followed by blocking with 1% BSA-1X PBS (w/v). The cells were then incubated with anti-Ki67 antibody [1:100 in 1% BSA-0.1% triton X-100- 1X PBS (w/v/v); cat. no. sc-15402, Santa Cruz Biotechnology, Inc.], overnight at 4°C, followed by incubation with secondary antibody (Alexa Fluor 488 goat anti-rabbit, 1:1,000 in 1% BSA-0.1% triton x100-PBS; cat. no. A-11078, Invitrogen; Thermo Fisher Scientific, Inc.), for 2 h at room temperature. Slides were mounted in VECTASHIELD media with DAPI (4'-6-diamidino-2-phenylindole; H-1200, Vector Laboratories, Inc.), and examined by standard fluorescence microscopy using an Axio Imager. Z1 microscope (Zeiss AG) with CytoVision[®] software version 3.9 and analyzed using ImageJ software MacOS X, with Java 1.8.0_172 (National Institutes of Health).

Wound healing assay. Cells were plated in 24-well plates (1x10⁵ cells/well) until the formation of a confluent monolayer. The cells were then incubated with mitomycin-C (M4287, Sigma-Aldrich; Merck KGaA), an antimitotic agent, for 3 h. A linear scratch in each monolayer was created using a P200 pipette tip, creating a gap across the well diameter. The media (EBM-2 supplemented with EGM-2 SingleQuots Supplements, which include 2% FBS; CC4101A, Lonza Group, Ltd.) was replaced to remove debris and cells in suspension, and to expose cells to the experimental conditions. Bright-field images of each well at 0 and 10 h were acquired using an Olympus IX53 inverted microscope (Olympus Corporation) and images were analyzed and quantified using ImageJ software MacOS X, with Java 1.8.0_172 (National Institutes of Health).

Rat aortic rings sprouting assay. Aortas (thoracic and abdominal segments) were dissected from male Wistar rats (aortas were collected from 10-week-old rats; n=6; used as controls; the rats were not submitted to any experimental condition) in the scope of another project. The study was approved by the Ethics Committee at NOVA Medical School (Ref. 75/2019/CEFCM). The rats housing conditions, as well as anesthesia and euthanasia procedures were as previously described (44). After removing all extraneous fat, fibrotic tissue and vasa vasorum structures, the aorta was segmented into rings with a length of ~1 mm. The rings were transferred

to a Petri dish and incubated overnight in FBS-free culture medium at 37°C with 5% CO₂. On the following day, the rings were embedded in Matrigel in a 24-well plate with EBM-2, with or without 100 μM propranolol. The medium was refreshed every 3-4 days, with the sprouts becoming visible at 7-13 days. Representative images were acquired using an Olympus IX53 inverted microscope (Olympus Corporation) and the branch points (intersections between ECs) and number per area were counted using ImageJ software MacOS X, with Java 1.8.0_172 (National Institutes of Health). The density of vessel-like structure formation (branch points number/μm²) was calculated as the proxy of vascular density.

Statistical analysis. All data were analyzed using a Student's t-test or one-way ANOVA and Tukey's post hoc test, in GraphPad Prism v7 software (www.graphpad.com/). The assays were performed with at least three biological replicates per condition. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical case presentation. A 13-year-old Caucasian girl presented complex partial seizures at the age of 18 months. An MRI scan revealed >30 brain lesions, some with evidence of recent bleeding, compatible with cavernomas (lesions were of several sizes, three with a diameter >10 mm, mainly hemispheric, in the cortical and subcortical regions). Apart from this, she had no relevant previous personal or family medical history. Her parents' imaging analyses did not reveal any notable vascular lesions.

At the time of diagnosis, she underwent surgery and a bleeding frontal lesion was partially resected; the pathology report confirmed a cavernoma lesion. Despite treatment with anti-epileptic drugs, the seizures recurred, usually at the same time each year. No causal association was established with the bleeding of the lesions, apart from a single time when bleeding and perilesional edema were documented in one cavernoma. She had no targeted therapy for cavernoma prior to her condition being brought to our attention.

She was examined for the first time in the aforementioned department at the age of 6 years. Following an examination, no notable neurological deficits were observed. She was under valproic acid and carbamazepine treatment. Genetic analysis revealed the presence of a CCM3-PDCD-10 mutation, one of the loci associated with CCMs (45,46). Propranolol therapy was commenced at the dose of 0.16 mg/kg/day and titrated to a maximum of 20 mg three times a day (0.8 mg/kg/day). At 6 years of follow-up, treatment with propranolol was well-tolerated and the seizures were controlled with valproic acid. Accordingly, vascular lesions were more exuberant before propranolol treatment (Fig. 1A), and MRI scans over the years revealed the spontaneous involution of some lesions and the stability of the others, without new bleeding events, as observed following a 6-year follow-up period (Fig. 1B).

Cellular and molecular effects. In the PB of the child patient with CCM prior to propranolol administration, the percentage of double-positive CD14⁺/CD31⁺ cells was higher than that in PB from healthy blood donors (Fig. 2A and B). Of note,

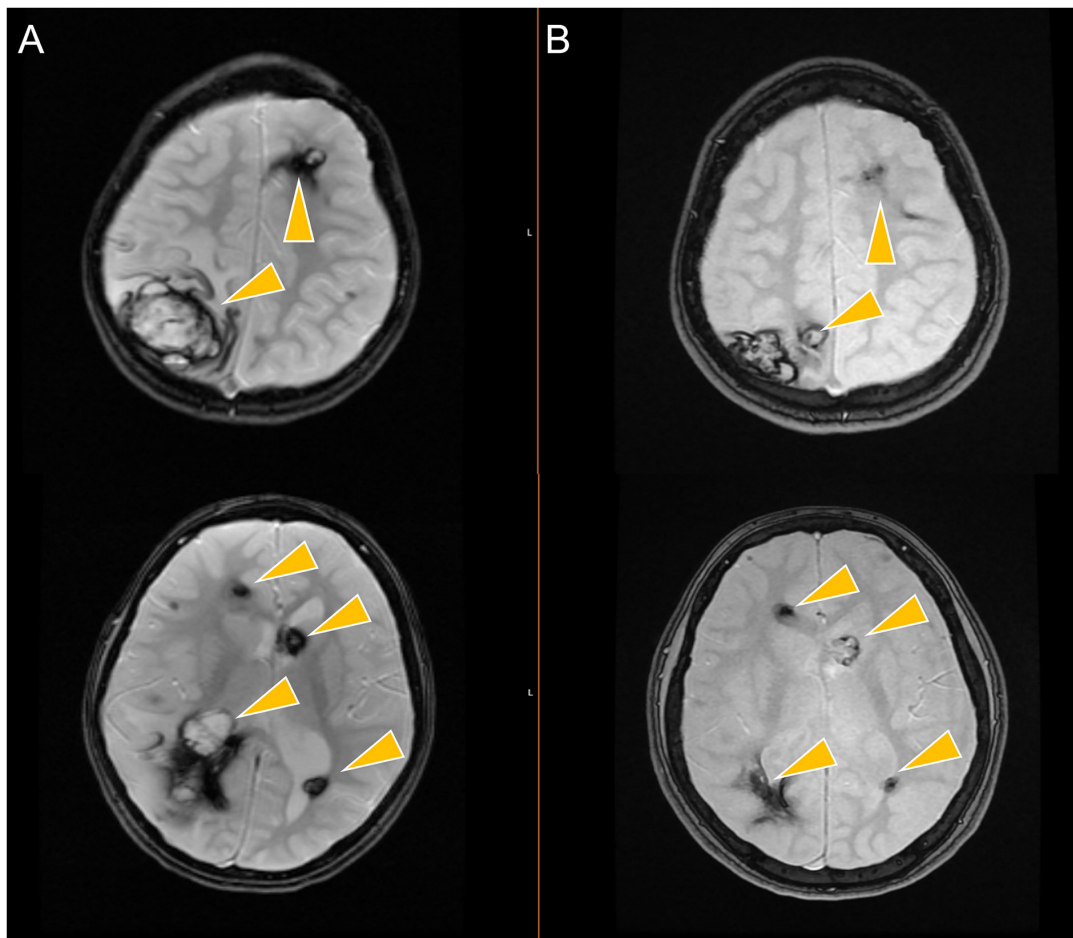


Figure 1. MRI illustrating a decrease in cavernoma structures in a child treated with propranolol for 6 years. MRI involved axial gradient-echo sequence. (A) Two sequential sections of multiple cavernomas with recent hemorrhage and mass effect, prior to propranolol therapy. (B) Involution of known cavernomas under propranolol treatment and no new lesions, under propranolol therapy (6 years of follow-up). Propranolol therapy was commenced at the dose of 0,16 mg/kg/day and titrated to a maximum of 20 mg three times a day (0.8 mg/kg/day). Control MRIs over the years revealed the spontaneous involution of some lesions and the stability of others (arrowheads), without new bleeding events. MRI, magnetic resonance imaging.

during the follow-up period with propranolol administration, a decrease in CD14⁺/CD31⁺ levels in PB was observed, with the levels being similar to those of the normal controls (Fig. 2A and B).

The concentration of VEGF in PB serum and in the culture medium conditioned by monocytes, isolated as previously described (27), was evaluated using ELISA. PB serum from healthy blood donors was used as normal controls and for cell supernatants, cells under control conditions were maintained in H₂O₂ and propranolol-free media.

The levels of VEGF in PB were higher prior to treatment with propranolol and decreased towards normal levels during follow-up (Fig. 2C). As regards monocytes exposed to H₂O₂, it was found that ROS decreased the VEGF levels in the culture media; however, a long exposure time to propranolol reverted this tendency (Fig. 3). Possibly, upon ROS generation, monocytes undergo an EC differentiation route and during this process, they lose the capacity of producing VEGF. Thereafter, the exposure of monocytes to propranolol increased VEGF production and accumulation in the culture medium.

The effects of propranolol on EC properties, such as proliferation (percentage Ki67⁺ nuclei/total nuclei) and migration

(wound healing assay) were evaluated *in vitro* using HUVECs. Propranolol (100 μ M) impaired EC angiogenic properties through a decrease in EC proliferation (Fig. 4A) and migration (Fig. 4B). The *ex vivo* effects of propranolol on EC activation and further vessel-like structures formation were evaluated using the rat aortic ring sprouting assay, in which it was proven that propranolol completely abrogated EC sprouting (Fig. 4C and D).

Discussion

Propranolol is a non-selective β -adrenergic blocker commonly used in the control of anxiety and cardiovascular conditions, such as hypertension, myocardial infarction and angina pectoris. Over the past decade, propranolol was re-discovered as an effective drug in the treatment of certain vascular tumors, inducing the rapid involution to quiescent residual lesions in 80% of cases (12,47-50). Its use in the treatment of infantile hemangiomas, the most common benign tumor of the skin, has been discovered accidentally and it was verified that propranolol administration is highly efficient in inducing tumor regression with very few adverse effects (40). Thus far, the beneficial effects of propranolol have been observed in

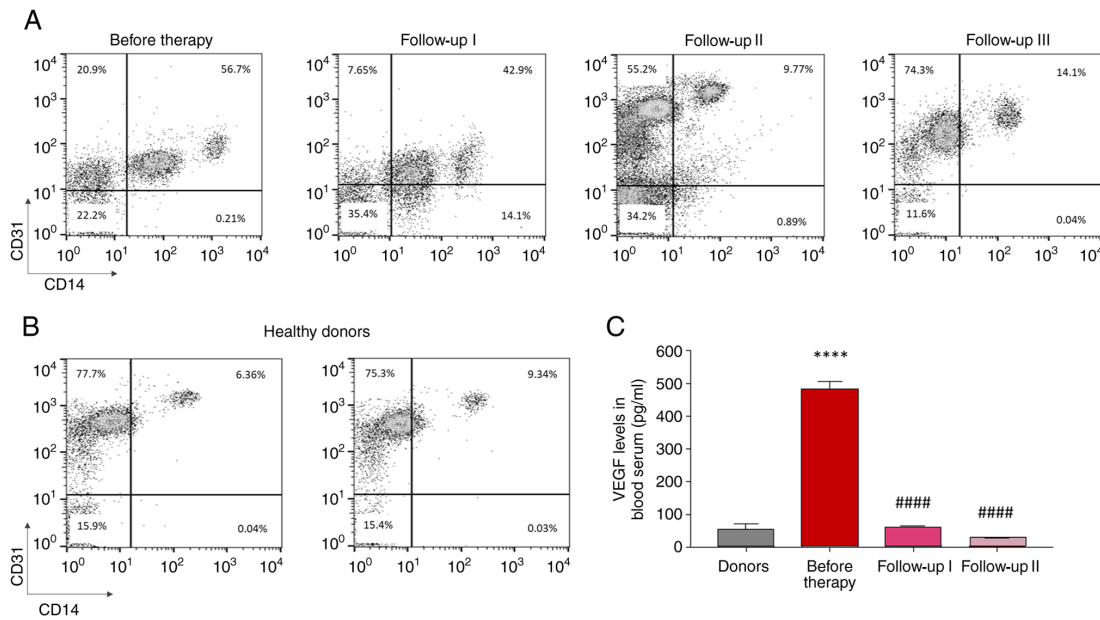


Figure 2. Percentage of CD14⁺ and CD31⁺ cells and VEGF levels in the PB of a child patient with a brain cavernoma decrease following propranolol therapy. (A) FACS analysis for CD14 and CD31 markers in total leukocytes. (B) FACS analysis for CD14 and CD31 in healthy donors. Cell labeling for CD14 and CD31 was performed as previously described by Lopes-Coelho *et al* (27). Before immunolabelling for FACS analysis, peripheral blood mononuclear cells were isolated from blood samples using Histopaque-1077. (C) ELISA for the measurement of VEGF levels in PB serum, using a VEGF Human ELISA kit. Data were analyzed using ANOVA with Tukey's post hoc test, with GraphPad Prism v7 software. ****P<0.0001, statistically significant difference vs. the healthy donors. ####P<0.0001, statistically significant difference between before treatment vs. Follow-up I and Follow-up II. PB, peripheral blood; VEGF, vascular endothelial growth factor; Prop, propranolol.

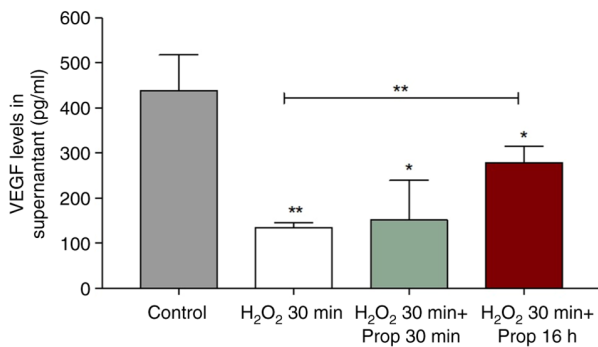


Figure 3. Monocytes exposed to reactive oxygen species (H₂O₂) exhibit a decreased production of VEGF, and propranolol exposure (16 h) partially reverses this effect. VEGF levels in the culture medium of cultured monocytes isolated from healthy donors exposed to H₂O₂ (15 μ M) and in the presence and/or absence of propranolol (100 μ M), were measured using the VEGF Human ELISA kit. Data were analyzed using ANOVA test with Tukey's post hoc test, with GraphPad Prism v7 software. The assays were performed with at least three biological replicates per condition. *P<0.05 and **P<0.01, statistically significant difference between control vs. H₂O₂, H₂O₂ + Prop (30 min) and H₂O₂ + Prop (16 h), and between H₂O₂ vs. H₂O₂ + Prop (30 min) and H₂O₂ + Prop (16 h). Prop, propranolol.

the treatment of neonatal hemangiomas (51,52), placental chorioangioma (53) and CCM (13).

In the present study, the administration of propranolol decreased the percentage of double-positive CD14⁺/CD31⁺ cells (monocytes) in the PB of the patient with CCM, reaching the levels presented by healthy blood donors during the follow-up period (Fig. 2A and B). The observed normalization of the CD14⁺/CD31⁺ cell levels upon propranolol administration suggested that the levels of circulating cells, sharing mono-

cytic and EC features, are involved in CCM pathogenesis and are propranolol-sensitive. Moreover, it was hypothesized that circulating monocytes sharing EC features (CD14⁺/CD31⁺) function as EPCs, as was recently described (27), contributing to CCM progression by being incorporated into CCM neovessels.

The exact mechanisms through which propranolol interferes with angiogenesis are not yet known; however, some studies have indicated that its anti-angiogenic effects are mediated by the downregulation of VEGF and FGF levels (12,40-42). The dynamics of VEGF were also addressed in the present study, in an attempt to clarify whether the VEGF levels are linked to CCM regression. In the patient described herein, a decrease in the levels of VEGF in the PB was observed upon propranolol treatment (Fig. 2C); the levels were similar to the values observed in healthy donors (Fig. 2C). Notably, *in vitro*, monocytes appear to use more VEGF upon H₂O₂ exposure, decreasing its free levels in conditioned culture medium; however, a longer exposure to propranolol, rescued the observed decrease in VEGF levels due to H₂O₂ exposure (Fig. 3). This observation suggests that propranolol, apart from affecting the levels of circulating VEGF, can also affect the way monocytes use VEGF *in vitro*, thus decreasing the overall pro-angiogenic capacity. According to the decreased stimulation of monocyte differentiation into ECs, it was also observed that propranolol affected the proliferation (Fig. 4A) and migration (Fig. 4B) of mature ECs. These observations are in agreement with recently published data by the authors demonstrating that propranolol also impairs vessel-like structure formation by ECs (43). Accordingly, the exposure to propranolol disrupted vessel-like sprouting in aortic rings (Fig. 4C and D). Since the VEGF levels may also be involved in

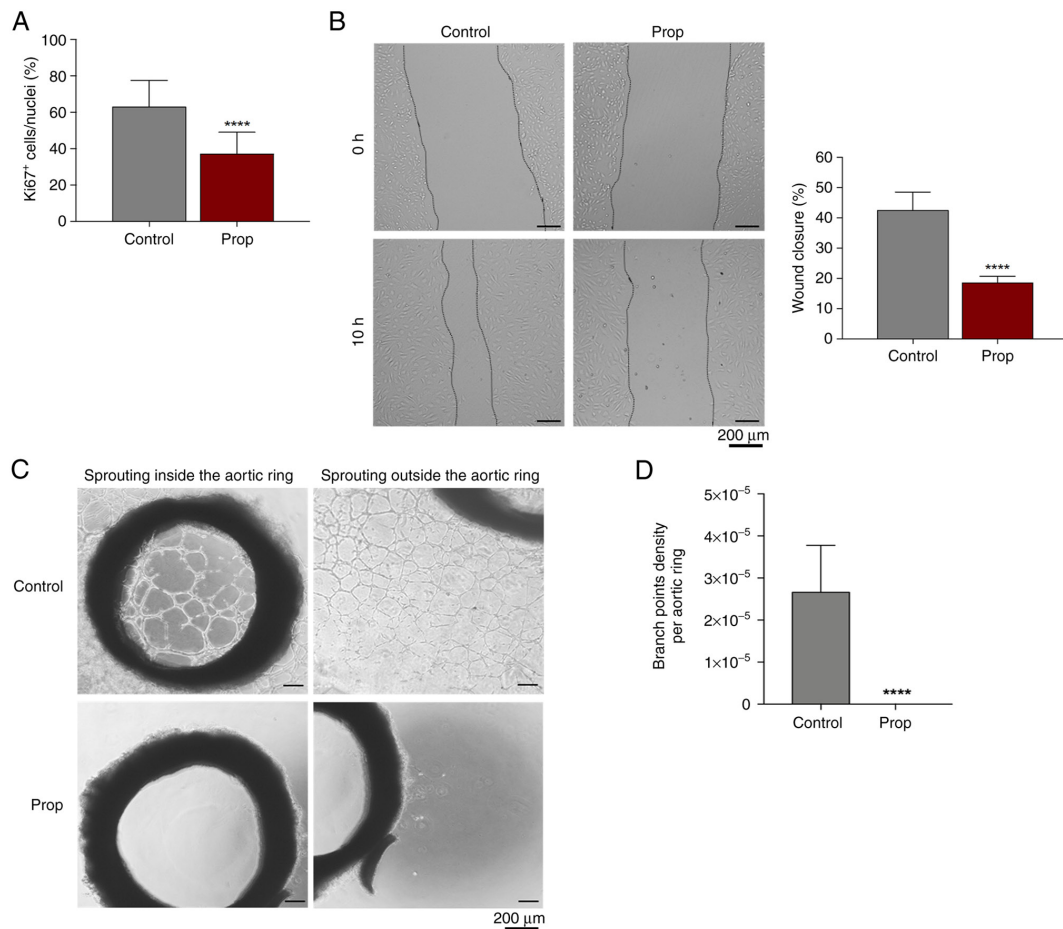


Figure 4. Propranolol decreases endothelial cell proliferation and migration, and impairs the capacity to form vessel-like structures. (A) Proliferation analysis based on the percentage of Ki67⁺ nuclei/total nuclei of HUVECs cultured with and without Prop (100 μ M), for 16 h. (B) Migration rate of HUVECs, previously exposed to mitomycin-C (3 h, 5 μ g/ml) to inhibit cell proliferation, in the absence and in presence of Prop, at time 0 h and after 10 h (C) Representative images at day 13 of aortic ring sprouting assay and the quantification of branch points density (D), in the presence or absence of propranolol. Assays with HUVECs were performed as previously described by Lopes-Coelho *et al* (43). Aortic rings sprouting assay was developed with aortas (thoracic and abdominal segments) dissected from male Wistar rats (10 weeks old) and cleaned to remove external tissue. After removing all extraneous fat, fibrotic tissue and vasa vasorum structures, the aorta was segmented into rings with a length of approximately 1 mm. For fluorescence and bright field microscopy, representative images were acquired using an Olympus IX53 inverted microscope. The quantification of Ki67⁺ nuclei, wound healing area and aortic rings branch points (intersections between ECs) was performed using ImageJ software. All data were analyzed using the Student's t-test with GraphPad Prism v7 software. The assays were performed with at least three biological replicates per condition. ****P<0.0001, statistically significant difference vs. control. Prop, propranolol; HUVECs, human umbilical vein endothelial cells.

monocyte recruitment (54-56), the decreased levels of VEGF, upon propranolol treatment, may be responsible, at least in part, by the decrease in the levels of circulating CD14⁺/CD31⁺ cells and CCM regression. However, further studies are required to elucidate the mechanisms through which propranolol affects VEGF dynamics in monocytes and ECs.

In zebrafish, the lack of CCM1, 2 or 3 constitutes them a reliable CCM animal model, since it results in abnormal EC sprouting and thin-walled vessels (46,57). Therefore, through a murine and an embryonic zebrafish model, Li *et al* (58) demonstrated that propranolol ameliorated cavernous malformation, possibly through the inhibition of β 1-adrenergic receptor, once the silencing of this receptor prevented vascular abnormalities. Additionally, several research groups have already demonstrated that VEGF levels are regulated by the catecholamines' pathway, since its levels are proportional to β -adrenergic receptors expression and can be inhibited by β -adrenergic receptor antagonists (59-61). For instance, melanoma cell lines exposed to norepinephrine, an adre-

nergic receptor agonist, have been shown to exhibit increased VEGF levels (62). However, other studies have yielded contradictory results, demonstrating that the anti-angiogenic effect of propranolol is independent of its β -blocker action. Sasaki *et al* (63) demonstrated that both β blockade by active S(-)- and inactive R(+)-propranolol enantiomers were able to downregulate the expression of angiopoietin like 4, an angiogenesis regulator, leading to the impairment of hemangioma growth *in vitro*. In fact, besides its effects on differentiated cells, Seebauer *et al* (64) demonstrated that the treatment of a murine xenograft model with the R(+) enantiomer inhibited the differentiation of hemangioma stem cells to ECs and further vessel formation. Moreover, recently, the authors demonstrated that propranolol exerted an anti-angiogenic effect through an antioxidant mechanism accounting for the inhibition of a ferroptosis-like mechanism, which in turn, impaired EC activation and the formation of vessel-like structures (43). Therefore, propranolol may present diverse mechanisms of action to impair vascular growth.

In summary, propranolol, apart from promoting the regression of CCM, impairs CD14⁺/CD31⁺ cell circulation (Fig. 2A), in part by the decreased VEGF levels (Fig. 2C). It was also observed *in vitro*, that stimulation with a prooxidant (H₂O₂) tended to promote the differentiation of monocytes in cell culture medium towards ECs (27), with decreased levels of VEGF. The underlying mechanism may involve the control of monocyte differentiation into ECs and how these cells are phenotypically altered. As recently demonstrated by the authors, oxidative stress promotes monocyte differentiation into ECs, and this process is reversed by propranolol, which appears to attenuate oxidative stress (43). Furthermore, VEGF is essential during the differentiation of monocytes into ECs (27); however, when monocytes differentiate into macrophage-like cells, they become VEGF-producing cells (56,65). This switch from macrophages to ECs explains the dynamics of VEGF in cell culture media. Considering that monocytes functioning as EPCs may favor the development of CCM lesions and given that VEGF is pivotal for monocytic differentiation into ECs, the increased circulating levels of VEGF observed in the patient with CCM without treatment may be crucial for potentiating the EC differentiation route and further, for preventing CCM pathogenesis.

Although the propranolol mechanisms of action in CCM are not yet fully understood, the lack of a better therapeutic option for patients with surgically inaccessible CCM and the notable responses in a few patients suggest that it may be of value to explore the exact efficacy of propranolol in the treatment of CCM, as well as the associated adverse side-effects (12). In accordance with this, randomized prospective clinical trials with propranolol vs. placebo/nothing groups [phase 1 trial NCT03523650, phase 2 trials NCT03474614 and NCT03589014 (66)] are currently ongoing. The findings of the present study reinforce the use of propranolol in the clinical management of CCM and points out the monitorization levels of monocytes (CD14⁺/CD31⁺) and VEGF in PB as useful tools which may be used to predict treatment efficacy.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

All authors (FLC, SN, FM, AH, SGF, GD, BFM, JFS, SVC, SAP, SV, DS and JS) participated in the conception and design of the study, and read and discussed the submitted and the accepted for publication versions of the manuscript. In addition, the authors contributed clinically and technically in the different stages of the study. FLC performed the analysis of the patient samples analysis, the analysis of the *in vitro* and *ex vivo* experiments, and prepared the first draft of the manuscript. FM, AH, SGF and GD performed the *in vitro* experiments. BFM and JFS performed the *ex vivo* experiments. SN, SV and DS were responsible for the clinical management of the patient. SAP and SVC coordinated the *in vitro* and *ex vivo* experiments. JS coordinated the whole project and was responsible for funding acquisition. FLC and JS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The PB of the child patient with cerebral cavernous malformation was collected after obtaining informed consent from the parents at the Neuropediatrics Department at Portuguese Institute of Oncology of Lisboa, Francisco Gentil (IPOLFG; ethics approval was obtained from the IPOLFG Ethics Committee; UIC-1137). Monocytes were isolated from PB collected after obtaining consent from healthy donors at Immuno-Hemotherapy Department at Portuguese Institute of Oncology of Lisboa, Francisco Gentil (IPOLFG). Ethics approval was obtained from the IPOLFG Ethics Committee; UIC-1137). The use of animals was approved by the Ethics Committee at NOVA Medical School (Ref. 75/2019/CEFCM).

Patient consent for publication

The consent from parents of the patient with cerebral cavernous malformation was obtained stating that they agreed for the data of their child to be published.

Competing interests

The authors declare that they have no competing interests.

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