Pediatric RESEARCH

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CLINICAL RESEARCH ARTICLE

Infantile hemangiomas β_3-adrenoceptor overexpression is associated with nonresponse to propranolol

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BACKGROUND: Propranolol (antagonist of β_1-/β_2-AR but minimally active against β_3-AR) is currently the first-line treatment for infantile hemangiomas (IH). Its efficacy is attributed to the blockade of β_2-AR. However, its success rate is ~60%. Considering the growing interest in the angiogenic role of β_3-ARs, we evaluated a possible relationship between β_3-AR expression and response to propranolol.

METHODS: Fifteen samples of surgical biopsies were collected from patients with IH. Three were taken precociously from infants and then successfully treated with propranolol (responder group). Twelve were taken later, from residual lesions noncompletely responsive to propranolol (nonresponder group). A morphometrical analysis of the percentage of β_1-, β_2-, and β_3-ARs positively stained area was compared between the two groups.

RESULTS: While no difference was found in both β_1- and β_2-AR expression level, a statistically significant increase of β_3-AR positively stained area was observed in the nonresponder group.

CONCLUSIONS: Although the number of biopsies is insufficient to draw definitive conclusions, and the different β-AR pattern may be theoretically explained by the different timing of samplings, this study suggests a possible correlation between β_3-AR expression and the reduced responsiveness to propranolol treatment. This study could pave the way for new therapeutic perspectives to manage IH.

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IMPACT:

- Propranolol (unselective antagonist of β_1 and β_2-ARs) is currently the first-line treatment for IHs, with a success rate of ~60%.
- Its effectiveness has been attributed to its ability to block β_2-ARs.
- However, β_3-ARs (on which propranolol is minimally active) were significantly more expressed in hemangioma biopsies taken from patients nonresponsive to propranolol.
- This study suggests a possible role of β_3-ARs in hemangioma pathogenesis and a possible new therapeutic target.

INTRODUCTION

Infantile hemangiomas (IH) are the most common soft-tissue tumors of infancy, affecting ~5% of Caucasian infants.[1] Despite the fact that IH usually tends to involute spontaneously, pharmacologic treatment is imperative when life-threatening complications, functional impairments, or disfigurements are observed or suspected.[2]

Since 2008, clinical studies reported the rapid regression of IH after treatment with propranolol,[3] a nonselective β_1/β_2-adrenergic receptor (β-AR) antagonist,[4] suggesting a profound and direct relationship between IH pathogenesis and the β-adrenergic system. The effectiveness of propranolol in counteracting IH growth has been attributed mainly to its ability to antagonize β_2- rather than β_1-ARs.[5] Currently, propranolol is considered as first-line treatment for IH, in view of its favorable risk–benefit ratio.[6] However, the success rate of this treatment (considered as a complete or nearly complete involution of IH) is of ~60%.[7] Moreover, severe sequelae are observed in >70% of infants treated with propranolol, and surgery is still needed in more than one-third of patients.[8] Therefore, it is likely that, in addition to β_1- and β_2-ARs, other receptors and pathways are involved in IH pathogenesis. The identification of these additional protagonists may lead to an improvement in therapeutic success.

The advent of propranolol for the treatment of IH led to a growing interest toward the pathogenesis of these tumors, and one of the most relevant novelties is the comprehension of the role played by hypoxia. The frequent detection of an ischemic "precursor mark" before the development of IH,[9] the frequent association with arterial anomalies ipsilateral to IH,[10] and the strong correlation with placental anomalies[11] suggests a

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relationship between regional hypoxia and IH development.[9,12,13] Hypoxia is currently recognized as the unifying pathogenic factor of apparently different pathologies such as IH, cancer, and retinopathy of prematurity, because all are characterized by the presence of hypoxia-induced angiogenesis.[14]

In all these pathologies, in addition to β_1- and β_2-ARs, an enormous interest has developed regarding the possible angiogenic role of β_3-ARs.[14–17] Interestingly, β_3-ARs, but not β_1- and β_2-ARs, are the only β-AR induced by hypoxia both in retina[14,16,18] and in cancer or tumor microenvironment,[15,17,19] where they are able to induce the upregulation of angiogenic factors such as vascular endothelial growth factor (VEGF), promoting the activation of endothelial nitric oxide synthase pathway.[14–16,20] Intriguingly, all three subtypes of β-ARs have been found in each evolutive phase (proliferating, late proliferating/early involuting, and involuting phase) of IH,[21] and β_3-ARs are the most expressed β-AR subtype in hemangioma-derived stem cells.[22]

Therefore, it cannot be excluded that the variable response to propranolol treatment (effective antagonist of β_1- and β_2-ARs but only minimally effective in blocking β_3-ARs)[23] might be related to a different pattern of β-AR expression. More specifically, we hypothesized that IH not responsive to propranolol might exhibit an increased expression of β_3-ARs compared to responsive IH.[14,24] To verify this hypothesis, we evaluated on histologic samples of IH with different response to propranolol treatment, the expression of β-ARs, and in particular of the β_3-AR.

METHODS

Patient eligibility and enrollment

Eligibility criteria to participate in this study were a diagnosis of IH, the treatment with propranolol, and the availability of histologic samples of IH. Histologic samples of patients referred from 2010 to 2019 to the Meyer University Children's Hospital with a diagnosis of IH were collected. Surgical biopsies were performed or before to start any treatment in case of diagnostic uncertainty, or after the treatment with propranolol during the surgical correction of clinical sequelae.

The following information was collected from each patient's medical records: sex, age, gestational age at birth, medical history, IH characteristics (age of appearance, localization and type), treatment history (treatment before starting propranolol, details of propranolol administration, including age at onset, dose and duration, IH outcome under propranolol, side effects, and concurrent treatments).

Photographic documentation was taken at the diagnosis and during the follow-up visits to determinate the response to the therapy.

Patients were categorized into two specific groups: (1) *Responders*, if propranolol treatment was completely successful or leaving the classical residual teleangectasias, and no other therapy was needed; (2) *Nonresponders*, in case of patients who needed additional treatments or surgical correction of prominent vascular residual lesions.

Immunofluorescent reactions

Immunofluorescent reactions were performed to evaluate the protein expression level and the localization of β-ARs in the collected samples. The histological analysis was carried out as follows: samples were rapidly excised, fixed in buffered 4% formaldehyde for 24 h, and embedded in paraffin. Couples of consecutive histological sections, 5 µm thick, were cut and placed on two different slides, the first one upward and the second one downward, in order to expose the two complementary cutting surfaces and achieve corresponding histological details. The embedding and cutting processes were performed by the Pathological Anatomy Department of the Careggi University Hospital in Florence.

After the deparaffination process, the sections were boiled for 10 min in sodium citrate buffer (10 mM, pH 6.0, Bio-Optica, Milan, Italy) or treated for 20 min at 90 °C with Tris-EDTA buffer solution (1 mM, pH 9.0) for antigen retrieval. To minimize the tissue autofluorescence, the sections were incubated in 2 mg/ml glycine (AppliChem, Darmstadt, Germany) for 8 min. After incubation in 1.5% bovine serum albumin in phosphate-buffered saline, pH 7.4, for 20 min at room temperature, the sections were immunostained overnight at 4 °C with the primary antibodies shown in Supplementary Table 1. Immunoreactions were revealed by appropriate fluorochrome-conjugated (Alexa Fluor 488- or 568-conjugated) IgG (1 : 300, Jackson ImmunoResearch Europe Ltd, Cambridge House, UK) for 2 h at room temperature. After the first incubation as described above, the sections were reincubated with a diverse primary antibody and with the appropriate secondary antibody, following the same procedures. Counterstaining was performed with aqua-based mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI) (Fluoroshield™ with DAPI, Sigma-Aldrich, St. Louis, MO, USA). Negative controls were carried out by replacing the primary antibodies with nonimmune rabbit serum. Human epidermal and pulmonary tissues were used as positive controls for the immunofluorescent reaction.

Representative images were acquired by an Olympus BX63 microscope coupled to CellSens Dimension Imaging Software version 1.6 (Olympus) at ×20, ×40, and ×100 magnification.

Morphometrical analysis

Morphometrical evaluation of the percentage of β_1-, β_2-, and β_3-AR positively stained area was performed introducing a semi-quantitative ordinal scoring system. A value ranging from 0 to 5 was assigned to each section (0, negative; 1, <10% of the area with positive staining; 2, between 11 and 30% of the area with positive staining; 3, between 31 and 50% of the area with positive staining; 4, between 51 and 75% of the area with positive staining; 5, >76% of the area with positive staining). To ensure the scoring reliability and repeatability, the morphometrical evaluation was performed by two histologists (A.P. and P.N.), first independently and then in a consensus panel. Each observer randomly scored the samples twice, at least two weeks after the first observation, and blinded about the patient.

Statistical analysis

The data are expressed as the mean ± SEM. Statistical analysis was carried out using unpaired, nonparametric, Student's *t* test via Mann–Whitney test one-way analysis of variance followed by Student–Newman–Keuls multiple comparison test. A probability P value ≤0.05 was considered significant. GraphPad Prism 6.0 statistical program (GraphPad Software, San Diego, CA) was used for statistical analysis.

Ethical approval

The study was conducted in accordance with the recommendations of Good Clinical Practice and approved by the Ethics Committee of the Meyer University Children's Hospital.

RESULTS

Of the 732 patients referred in the period 2010–2019 to the Meyer University Children's Hospital with a diagnosis of IH, 111 were selected for propranolol treatment. We collected 15 histologic samples of surgical biopsy, the only available samples. Three of these 15 patients were subjected to a precocious, pretreatment, surgical biopsy for diagnostic uncertainty, while 12 patients underwent later biopsy on the occasion of a surgical correction of the posttreatment unesthetic sequelae.

Clinical features

Clinical features of patients are summarized in Table 1. A prevalence in the female sex can be appreciated (80%). Six

Table 1. Medical records of the patients and IH characteristics.

Patient code	Sex	Gestational age (weeks)	Age of appearance	Type	Subtype	Localization
1	Female	34 + 1	At birth	Superficial	Segmental	Face and gastrointestinal (PHACE syndrome)
2	Male	Term	First week	Mixed	Focal	Frontal
3	Female	31^{+2}	At birth	Deep	Focal	Latero-cervical
4	Female	Term	At birth	Mixed	Focal	Frontal
5	Female	35	Second week	Mixed	Focal	Periorbital
6	Female	Term	First week	Superficial	Focal	Right shoulder
7	Female	Term	First week	Mixed	Focal	Lower lip and laryngeal
8	Male	35^{+2}	First week	Mixed	Focal	Left nasal ala
9	Female	Term	First week	Mixed	Focal	Nasal-cyrano
10	Male	Term	Third week	Deep	Focal	Left eyelid
11	Female	Term	Second week	Superficial	Focal	Retro-auricular
12	Female	Term	Second week	Superficial	Segmental	Face (PHACE syndrome)
13	Male	33	First week	Mixed	Focal	Nasal-cyrano
14	Female	32	Second week	Mixed	Focal	Upper lip
15	Male	Term	First week	Mixed	Focal	Left shoulder

Table 2. Treatment history for propranolol and other therapies.

Patient code	Treatment before propranolol	Age at propranolol onset (months)	Max dosage	Duration (months)	Outcome	Treatment after propranolol	Age at surgery (months or years)	Concomitant therapies
1	No	1	3 mg/kg/day	2.5	Responder	No	–	
2	Steroids	6	3 mg/kg/day	9	Nonresponder	Surgery	3 yr	
3	No	1.5	3 mg/kg/day	6	Responder	No	–	
4	No	6	3 mg/kg/day	10	Nonresponder	Surgery	2.5 yr	
5	No	4	3 mg/kg/day	12	Nonresponder	Surgery	1.5 yr	Steroids (2 m)
6	No	2	3 mg/kg/day	8	Nonresponder	Surgery + dye laser	9 m	
7	No	2	3 mg/kg/day	15	Nonresponder	Surgery	4 yr	
8	Topical timolol	6	3 mg/kg/day	12	Nonresponder	Surgery	2.5 yr	
9	No	3.5	3 mg/kg/day	6	Nonresponder	Surgery	2 yr	
10	Topical timolol	7	3 mg/kg/day	9	Responder	No	–	
11	No	5	3 mg/kg/day	9	Nonresponder	Surgery	4 yr	
12	No	4	3 mg/kg/day	12	Nonresponder	Surgery	8 yr	Steroids (5 m)
13	No	3	3 mg/kg/day	10	Nonresponder	Surgery	3 yr	Steroids (2 m)
14	No	2.5	3 mg/kg/day	34	Nonresponder	Surgery	5 yr	
15	No	3	3 mg/kg/day	12	Nonresponder	Surgery	1.5 yr	

patients were preterm (40%). IH was categorized, following the classification of International Society for the Study of Vascular Anomalies (ISSVA),[25] in superficial (27%), deep (13%), and mixed (60%). IH subtypes observed were principally focal (87%) and only 13% were segmental. In most cases, IH appeared between the first and the second weeks of life, and only in two newborns, some precursor was present at birth. Most of the patients (86%) presented IH affecting the head and neck, with higher distribution in the face area: three in the nasal region, two in the orbital region, two in the frontal region, two on the lips, and two in the laterocervical and retroauricular region. Upper limb location was detected in two patients. PHACES syndrome (acronym describing a syndrome characterized by the association of posterior fossa brain malformations, large facial hemangiomas, anatomical anomalies of the cerebral arteries, aortic coarctation or other cardiac anomalies, and eye abnormalities)[26] was observed in two patients (13%): both presented a segmental IH of the face associated with anomalies such as coarctation of the aorta, right aortic arch, and ocular and neurological anomalies

All the patients underwent the same protocol of propranolol treatment: the initial dose was 1 mg/kg/day, with an increase of 1 mg/week up to the maximum dosage of 3 mg/kg/day, which was maintained for the whole treatment period. As shown in Table 2, treatment was started at a mean age of 3.5 ± 1.8 months and it was maintained for an average duration of 10 ± 7 months. During treatment, no significative adverse effects related to the propranolol therapy were reported. Three patients, after a treatment of 6 ± 3 months, showed a complete regression of the hemangioma, without the need of any other medical or surgical treatment. In these patients, the surgical biopsy was performed before to start propranolol because the diagnosis was uncertain. In all these patients, the detection of glucose transporter-1-positive stain confirmed the diagnosis of IH.[27] Twelve patients showed no response (five patients) or only partial response (seven

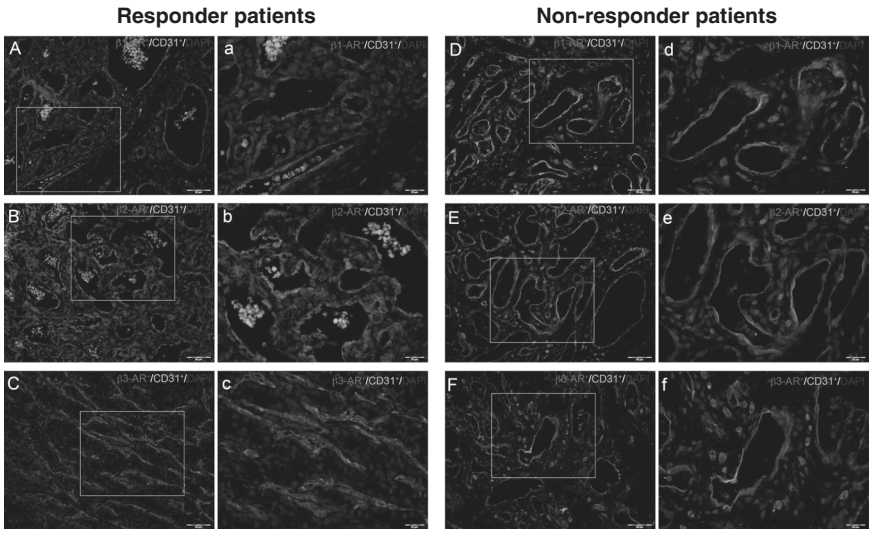

Fig. 1 β-AR expression profile in biopsies of responder and nonresponder patients. Representative images of β-AR (in red) and CD31 (in green) double labeling carried out on the HI samples of responder and nonresponder patients. In responder patients, β1-AR (**A**, **a**) and β2-AR expression (**B**, **b**) is present on endothelial cells, while β3-AR immunoreactivity (**C**, **c**) is weak or even undetectable. In nonresponder patients, β1-AR (**D**, **d**) and β2-AR signals (**E**, **e**) are mainly located on endothelial cells but are also present in scattered perivascular cells. β3-AR is widely expressed by endothelial cells and by numerous dispersed cells of neoplastic tissue (**F**, **f**). Nuclei are stained blue with DAPI. Scale bars are indicated in each panel.

patients) after 10 ± 2.5 months of treatment with propranolol: all needed a surgical removal of residual lesions, sometimes for esthetic disfiguration. Therefore, tissue samples of these patients were obtained by lesions that persisted after treatment. Surgery was performed at 3.5 ± 1.8 years of age, 6–18 months after the end of the pharmacologic treatment.

Morphological analysis of β-AR expression

The immunofluorescence analysis clearly demonstrated the presence of all the β-AR subtypes in most of the IH samples evaluated (Fig. 1), with some differences in the tissue localization and the positively stained area extent.

In particular, in the samples of responder patients, β_1- and β_2-AR expression was mainly located on tunica intima of the vascular wall, as demonstrated by their copresence with the endothelial cell marker, CD31 (Fig. 1A, a, B, b). In nonresponder patients, β1- and β2-AR immunoreactivities were also present in scattered cells, in neoplastic perivascular tissue (Fig. 1D, d, E, e). The expression profile of the β3-AR markedly differed between the two groups of patients. Its immunoreactivity was generally weak or even undetectable in the samples of patients responsive to propranolol (Fig. 1C, c), while it was widely expressed by endothelial cells (coexpressed with CD31) and by numerous dispersed cells of neoplastic tissue in the nonresponder patients (Fig. 1F, f).

To investigate the putative nature of the non-endothelial β_3-AR-positive cells, we performed double immunolabeling reactions on couple of histological sections. Some of β_3-AR-positive cells also expressed CD34 and CD133 (Fig. 2A, a, B, b), being identifiable as endothelial progenitor cells (EPCs).[28]

Positive controls of β_1-AR, β_3-AR, and β_2-AR antibodies were carried out performing immunofluorescence reactions on human lung and epidermal sections, respectively. According to data reported in the literature, β_1-AR and β_3-AR were expressed on bronchial epithelial cells[29] (Supplementary Fig. 1A, a, C, c, respectively) and β_2-ARs were present on keratinocytes[30] (Supplementary Fig. 1B, b).

Morphometrical analysis of β-AR expression

The immunofluorescent reactions were also subjected to semi-quantitative morphological analysis, according to the ordinal scoring system previously described. No statistically significative difference was found in both the β_1- and β_2-AR expression levels between the responder and the nonresponder groups (Fig. 3A, B). On the contrary, the scoring analysis revealed a statistically significant increase of β_3-AR positively stained area ($p \leq 0.05$) in the non-responder patients compared to the responder ones (Fig. 3). These data indicated a correlation between β_3-AR expression with/and the unresponsiveness of HI to propranolol treatment.

DISCUSSION

Despite the significant recent progresses in the treatment of IH, the mechanisms underlying their pathogenesis are not fully clarified yet. To date, the hypothesis of a somatic mutation in a precursor stem cell of a critical gene able to induce angiogenesis persists,[31] together with the hypothesis of an embolization of fetal tissues by placental cells,[32] as suggested by the increased risk of IH after chorionic villus sampling[33] and by the similarity in tissue-specific markers between placental vessels and IH.[34] Interestingly, hemangioma-derived multipotent stem cells are able to reproduce hemangioma-like lesions in immunodeficient mice, suggesting the origin of IH from progenitor cells.[35]

A great progress in understanding the pathogenesis of IH has originated from the identification of the role of hypoxia and the adrenergic system, able to recruit EPCs.[14] These cells are attracted toward hypoxic tissues via hypoxia-inducible factor 1, through the action of VEGF and stromal cell-derived factor 1α.[36] A very high number of circulating marrow-derived undifferentiated EPCs are detected in children with proliferating IH, suggesting that IH may originate from a process of vasculogenesis rather than of angiogenesis.[37,38] Indeed, vasculogenesis may result from the recruitment of EPCs induced by the binding of stromal cell-derived factor 1α to C-X-C chemokine receptor type 4, a receptor highly expressed in EPCs,[39] and the downstream activation of Akt and mitogen-activated protein kinase signaling pathways.[40] Therefore, EPC may represent a key cell in IH growth.

Even the mechanism of action of propranolol remains uncertain. Propranolol, predominantly active on β1- and β2-ARs, rapidly reduces redness and softens the tumor, and therefore it is reasonable to suppose that these effects are due to its well-known vasoconstrictive effects.[41]

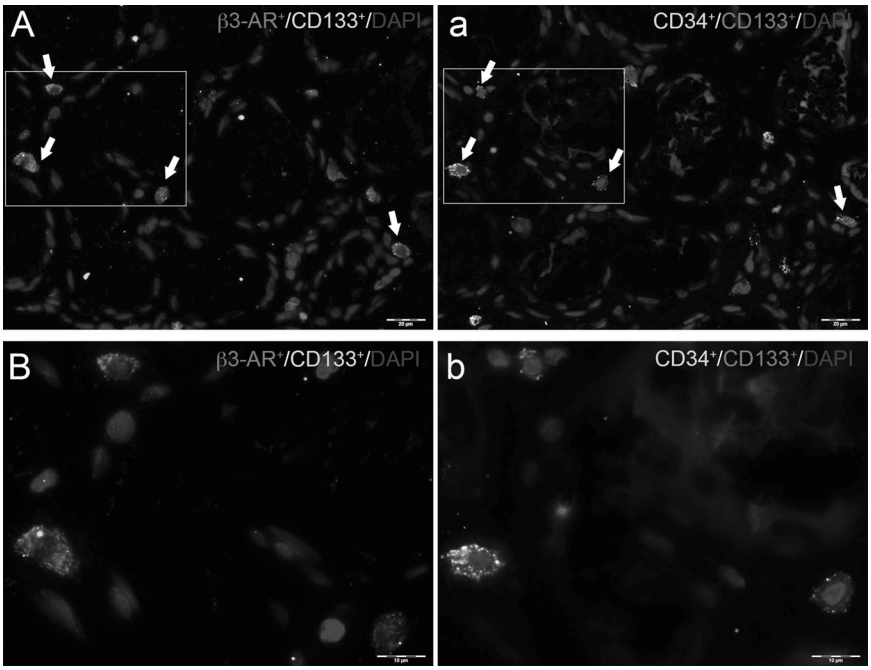

Fig. 2 β3-AR expression in endothelial progenitor cells. Representative images of double immunolabeling reactions performed on couple of histological sections. Panels **a** and **b** show the complementary cutting surface of the panels **A** and **B**, respectively, and white arrows indicate the same cells present in both the corresponding specular fields. As reported in **A**, **B**, some of the β3-AR-positive cells (in red) are also CD133-positive (in green). In **a**, **b**, the same β3-AR/CD133 (in red)-positive cells also express CD34 (in green). This couple of histological section shows β3-AR/CD133/CD34-positive cells, thus identifiable as EPCs. Nuclei are stained blue with DAPI. Scale bars are indicated in each panel.

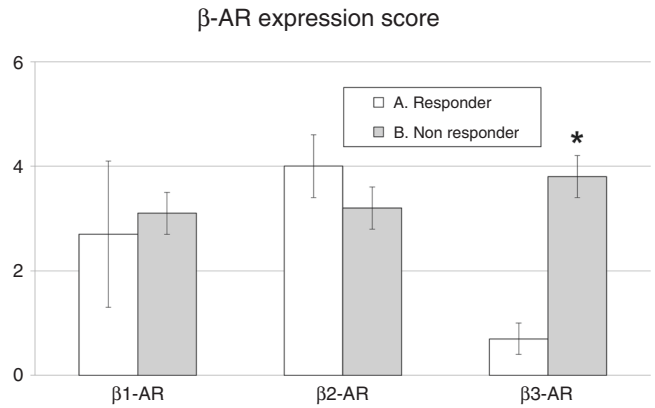

Fig. 3 Semiquantitative morphological analysis of β-AR expression profile. Semiquantitative analysis of β-AR expression performed on immunofluorescence reactions. No difference is revealed in both the β1-AR and β2-AR expression levels between the responder and the nonresponder groups (**A** and **B**, respectively). On the contrary, the morphometrical analysis shows a statistically significative increase ($*p \leq 0.05$) of β3-AR positively stained area of the nonresponder patients compared to the responder ones.

However, propranolol also inhibits viability and proliferation of hemangioma-derived endothelial cells by inducing both intrinsic and extrinsic apoptotic pathways,[42,43] and reducing the expression of proangiogenic factors. VEGF expression is downregulated by propranolol in a dose-dependent manner,[42] as confirmed by the significant reduction of serum proangiogenic factors in infants with IH after propranolol treatment.[44,45] The efficacy of propranolol is, at least in part, related to its effects on progenitor/stem cells, because in these cells, it downregulates the expression of both VEGF and basic fibroblast growth factor,[46] inhibits Akt and mitogen-activated protein kinase phosphorylation, and decreases C–X–C chemokine receptor type 4 expression, preventing the EPC homing.[40]

The effectiveness of propranolol has been attributed to its ability to block β_2-ARs rather than β_1-ARs. Indeed, in hemangioma endothelial cells, β_2-AR is the dominating β-AR,[47] and the increase of VEGF expression induced by isoproterenol, agonist of β_1- and β_2-ARs, is prevented only by the selective β_2-AR blocker ICI 118,551.[5]

However, in addition to β_2-AR, also β_3-AR seems to play a role in the pathogenesis of IH. Indeed, β_3-AR is expressed in the vascular wall, where it promotes the activation of endothelial nitric oxide synthase and consequent vasodilation,[20] and is recognizable in all phases of IH.[21] Interestingly, β_3-AR is the most expressed subtype in hemangioma-derived stem cells,[22] while it is not expressed by mature IH-derived endothelial cells.[48]

Considering the very low antagonistic activity of propranolol on β_3-ARs,[23] recently we launched the hypothesis that the different expression of this receptor might condition the therapeutic response to propranolol and that IH refractory to propranolol may have an increased expression of β_3-ARs,[14] the hypothesis recently reproposed.[24]

As shown in Table 3, the affinity of propranolol for β_3-ARs is significantly lower than that observed for β_1- or β_2-ARs. In fact, the inhibitory constant (K_i) of propranolol for β_3-ARs is ~200 times higher,[23] suggesting an affinity 200 times lower, than for β_1- or β_2-ARs.[49] At the same time, it is instead evident that the affinity of norepinephrine for β_3-ARs is similar to that for β1-ARs but significantly higher than that for β_2-ARs.[23] The analysis of Table 3 suggests that propranolol, at the plasma concentrations usually reached in the pediatric population,[50] is only minimally active against β_3-ARs. On the contrary, the typical release of norepinephrine in the microenvironment induced by tissue hypoxia,[14,18,51] together with its high affinity with β_3-ARs, suggests a possible pathogenetic involvement of this receptor in the persistence of vascular lesions.

Table 3. Binding affinities for the main physiologic agonists of β-AR subtypes and propranolol.[23]

	β1-AR K_i (nM) (95% CI)	β2-AR K_i (nM) (95% CI)	β3-AR K_i (nM) (95% CI)
Norepinephrine	3570 (2440–5210)	26,400 (23,400–29,900)	4300 (4240–4360)
Epinephrine	3970 (2840–5530)	735 (510–1050)	126,000 (116,000–136,000)
S-propranolol	1.8 (1.2–2.8)	0.8 (0.6–1.0)	186 (134–259)

Moreover, the present results showing a significantly higher expression of β_3-ARs in IH samples of patients nonresponsive to propranolol strengthen, even though it does not prove our hypothesis.

The high expression of β_3-ARs in samples nonresponsive to propranolol and on progenitor cells is not surprising and in line with our recent studies, performed in different animal and human models, such as in oxygen-induced retinopathy (OIR) or in cancer, respectively.

In OIR mice model, oxygen exposure induces an initial retinal ischemia, and a consequent progressive hypoxia that is responsible for the following retinal neovascularization, miming what happens in newborns with retinopathy of premature (ROP). In C57BL/6J mice, we have demonstrated that treatment with propranolol, similarly to what happens for IH, is able to reduce such neovascularization.[18,52] This efficacy has been confirmed also in human preterm infants with ROP.[53,54] However, in a different strain, the Sv129S6 mice, characterized by a much more aggressive OIR with a huge production of VEGF,[55] treatment with propranolol was ineffective.[56] Interestingly, during the development of such neovascularization, that is, during the hypoxic phase, an upregulation of β_3-ARs was noted in both strains, but their increase in the Sv129S6 strain was impressive.[56] This observation suggested us that the lack of efficacy of propranolol (only minimally active in blocking β_3-AR) in the strain with the strongest upregulation of β_3-ARs may be not accidental, and that β_3-ARs might play a crucial angiogenic role,[57] as strengthened by the observation that β_3-AR blockade inhibits the release of proangiogenic factors in retinal explants.[16]

In melanoma and in neuroblastoma, we recently confirmed the modulation of β_3-ARs induced by hypoxia and demonstrated a strong relationship between β_3-AR expression and stemness. In melanoma microenvironment, β_3-ARs were strongly expressed in cancer-associated fibroblasts, macrophages, endothelial cells, and EPC, and were actively involved in their recruitment. Moreover, β_3-ARs induced stem-like traits,[17] while their blockade induced their differentiation.[58] A similar effect was demonstrated in neuroblastoma, where β_3-AR antagonism switched neuroblastoma cells from stemness to cell differentiation.[59]

Therefore, this study suggests that the variable response to propranolol might be related to a different prevalence of β_3-AR, and that IH with higher expression of β_3-AR, mainly located on EPC, may be resistant to propranolol. If these results will be confirmed by further researches, a new therapeutic scenario could open up.

However, this study has potential limitations. First, the small number of enrolled patients. This is due to the fact that the management of IH is usually clinical, and biopsy is performed only in a minority of patients. Second, biopsy tissue samples were "non-symmetrically" collected. Indeed, all the samples of patients responsive to propranolol were collected precociously, to clarify a diagnostic uncertainty. Conversely, all the samples of nonresponsive patients were taken later, during a surgical procedure. This inhomogeneity of the samples certainly represents the point of greatest fragility of the study since we cannot exclude a priori that the expression of the β-ARs changes over time. However, it is very unlikely a possible relationship between the higher expression of β_3-ARs observed in samples of nonresponsive patients with their later collection, when it is logical to expect their reduced expression. In fact, considering that IH are lesions originally hypoxic that progressively normalize their oxygenation,[9] it is very unlikely that β_3-ARs, usually upregulated under hypoxia[14,16–19,56,60,61], are in this context increased over time during hypoxia resolution. Moreover, it is known that the normal evolution of IH is toward a fibroadipose transdifferentation, and recently we demonstrated that this process is even promoted by β_3-AR antagonism,[58] suggesting that IH regression is related with the downregulation/inactivation of β_3-ARs rather than their upregulation. On the contrary, these observations make it possible to speculate that β_3-AR upregulation within the samples of nonresponsive patients could be the consequence of a persistent hypoxia in the samples resistant to propranolol. Third, it is important to highlight that the "late" samples were collected after a treatment with propranolol lasting many months. Also, in this case, it is impossible to exclude a priori that a treatment with a drug able to block β_1- and β_2-ARs can induce an upregulation of β_3-ARs. However, this hypothesis seems an unlikely eventuality. Indeed, even in the extreme case of β_1- and β_2-AR-knockout mice, no compensatory β_3-AR upregulation was observed.[18] Moreover, overall, surgical procedures were performed months or even years after the treatment suspension. It is therefore improbable that the putative effects of propranolol on β_3-AR upregulation were still present. Another limitation of the study was the availability of only paraffin-embedded samples that strongly limited the feasible analyses. In the future, if nonfixed samples should be accessible, both ELISA and real-time PCR analysis could be performed to precisely quantify the protein and the mRNA expression level of the receptor in the tissue, respectively. Finally, at the present time there are no approved blockers for targeting β_3-ARs in humans, and this lack prevents us from evaluating their possible efficacy in human clinical trials.

In conclusion, this study suggests a possible involvement of β_3-ARs in the partial efficacy of treatment with propranolol. The confirmation of our findings by further studies could pave the way for a more effective therapy of IH.

AUTHOR CONTRIBUTIONS

C.F. conceptualized and designed the study, drafted the initial paper, and reviewed and revised the paper. A.B., C.F., T.O., and R.M.G. collected data, carried out the initial analyses, and reviewed and revised the paper. A.P., P.N., and C.C. embedded and cut biopsies, performed immunofluorescent reactions, morphometrical analysis, and reviewed and revised the paper. All authors approved the final paper as submitted and agree to be accountable for all aspects of the work.

ADDITIONAL INFORMATION

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