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Aqueous humor erythropoietin levels in open-angle glaucoma patients with and without TTR V30M familial amyloid polyneuropathy

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Purpose: Glaucoma is the leading cause of irreversible blindness in familial amyloidotic polyneuropathy (FAP) patients. Erythropoietin (EPO) is a cytokine that has been shown to play a role in neuroprotection and is endogenously produced in the eye. EPO levels in the aqueous humor are increased in eyes with glaucoma. In this study, we evaluated the EPO concentration in the aqueous humor of FAP and non-FAP patients, with and without glaucoma.
Methods: Undiluted aqueous humor samples were obtained from 42 eyes that underwent glaucoma surgery, phacoemulsification, or vitrectomy. EPO concentration in the aqueous humor and blood were measured using the Immulite 2000 Xpi using an automatic analyzer (Siemens Healthcare Diagnostics).
Results: The mean EPO concentration in the aqueous humor of non-FAP glaucoma eyes group 2 (75.73±13.25 mU/ml) was significantly higher than non-FAP cataract eyes (17.22±5.33 mU/ml; $p<0.001$), FAP glaucoma eyes (18.82±10.16 mU/ml; $p<0.001$), and FAP nonglaucoma eyes (20.62±6.22 mU/ml; $p<0.001$). There was no statistically significant difference between FAP nonglaucoma eyes versus non-FAP cataract eyes ($p = 0.23$) and FAP glaucoma eyes versus FAP nonglaucoma eyes ($p = 0.29$). In the glaucoma groups, there was no correlation between the aqueous humor EPO concentration and the ocular pressure ($p = 0.95$) and mean deviation ($p = 0.41$). There was no correlation between the EPO serum concentration and EPO aqueous humor concentration in our patients ($p = 0.77$).
Conclusions: Unlike other glaucomatous patients, FAP patients with glaucoma do not show increased and potentially neuroprotective endocular EPO production in the aqueous humor and may need more aggressive glaucoma management.

Glaucoma is a progressive optic nerve neuropathy and the major cause of preventable and irreversible blindness worldwide. It is characterized by visual field defects and nerve head cupping due to the loss of retinal ganglion cells [1]. Despite its multifactorial genesis [2-4], the major risk factor for glaucoma progression is the elevated intraocular pressure (IOP) [5,6], which compresses the retinal ganglion cells at the optic nerve head [7]. The only treatment that slows glaucoma progression involves lowering the IOP [8].

Familial amyloid polyneuropathy (FAP) is caused by the extracellular deposition of amyloid fibrils of mutant transthyretin (TTR) V30M in various tissues and organs [9-11]. TTR V30M mutation is the most common form of transthyretin amyloidosis (ATTR) variant in Portugal as well as in the world [12]. The main clinical expression of FAP disease is a sensorimotor and autonomic neuropathy, but other manifestations, such as nephropathy and hematologic and ocular abnormalities can occur. Among the reported ocular FAP complications [13-15], glaucoma is the major cause of irreversible vision loss and is often difficult to control [16].

Erythropoietin (EPO) was identified as a hematopoietic cytokine that promotes proerythroblast survival and maturation [17]. Recently, EPO was recognized as a member of the cytokine type 1 superfamily with multiple functions outside the bone marrow [18]. It provides direct protection against hypoxia by its anti-apoptotic, anti-oxidative, and anti-inflammatory properties and for its angiogenic capacity that allows the oxygen supply to ischemic tissues. Several studies have found that EPO protects photoreceptor cells, retinal ganglion cells, and retinal pigment epithelial cells from apoptosis [19-26]. Hernandez et al. [27] suggested that EPO is produced locally in the retina. Muller cells and retinal pigment epithelium were identified by Fu et al. [28] and Garcia-Ramírez et al. [29], respectively, as the cells responsible for EPO production in the eye.

Previous studies have shown a significantly increased EPO concentration in the aqueous humor of eyes with glaucoma [30-32]; this is probably a defence mechanism against

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glaucomatous damage [33] caused by hypoxia, ischemia, oxidative stress, and reduced pro-inflammatory cytokine production [34-39]. Although hypoxia/ischemia is the major stimulus for endocular and systemic EPO production [21,40-43], other incompletely understood factors may be involved [27,29].

FAP patients and even presymptomatic carriers have an inappropriately low EPO production [44]. In vitro studies suggest that the dissociated mutant TTR that polymerizes into misfolding amyloidogenic intermediates, protofilaments, and nonfibrillar aggregates of TTR rather than mature amyloid fibrils may induce cellular toxicity [45,46]. We propose that these amyloid precursors may be toxic to EPO-producing cells. This study was performed to evaluate the ocular EPO response in FAP patients with glaucoma.

METHODS

It was recruited 42 eyes of 42 patients (18 females) with a mean age of 56.8±7.4 years. A prospective, controlled, nonrandomized, nonblind comparative study was conducted from January 2008 to December 2011 at the Ophthalmic and Clinical Chemistry Departments from Centro Hospitalar do Porto, Porto. Written informed consent was obtained from all patients. This study was performed in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Ethics Committee of the Centro Hospitalar do Porto.

Presurgical assessment included Snellen best-corrected visual acuity (Snellen chart, Takagi chart projector CP-30, calibrated for approximately 6 m), slit-lamp biomicroscopy, intraocular pressure (IOP) measurement by Goldman applanation tonometry (same person with AT-900 tonometer; Haag-Streit, Koniz, Switzerland), fundoscopy (90 D noncontact slit-lamp lens; Volk Optical, Mentor, OH), Humphrey perimetry (Humphrey Field Analyzer; Humphrey Instruments, San Leandro, CA), and the cup/disc ratio. All examinations were performed within 2 weeks before the surgical procedure.

Exclusion criteria for all groups were: previous laser and/or intraocular surgery; history of systemic (e.g., diabetes mellitus, kidney disease, cardiovascular disorders, anemia, immune disease, except FAP in groups 1 and 3) or any ocular disorders (e.g., age-related macular degeneration); history of medications that could influence the level of EPO (e.g., iron preparations, chemotherapeutic agents, granulocyte colony-stimulating factor, or systemic therapy with EPO), and patients with any type of glaucoma except open-angle glaucoma, such as angle-closure, pigmented, exfoliation, normotensive, and neovascular glaucomas, or ocular hypertension.

To clarify the relationship between aqueous EPO production and circulating blood EPO levels, we compared the aqueous and serum concentrations of EPO. Aqueous humor samples were obtained from each eye before the beginning of surgery (trabeculectomy, phacoemulsification, or vitrectomy). The standard procedure involved collecting undiluted aqueous humor samples (50–150 µl) through a paracentesis, using a 30-gauge needle on a tuberculin syringe under an operating microscope. Samples were obtained carefully to avoid touching intraocular tissues or blood contamination. All samples were carefully protected from light and were sent immediately to the laboratory for EPO measurement. At the same time, 9 ml of venous blood samples were collected in EDTA tubes from an antecubital vein immediately before sugery. The blood was immediately centrifuged and the blood serum put on the automatic analyzer.

Serum samples were obtained from the centrifugation of the blood sample. The samples of aqueous humor and serum had the same processing routine analysis. Serum and aqueous humor EPO concentrations were measured by a chemiluminescent method in an automatic Xpi Immulite 2000 analyzer (Siemens Healthcare Diagnostics, Siemens AG, Munich, Germany).

Statistical analysis: Statistical analysis was performed using nonparametric tests. The Kruskal–Wallis test was used to compare the groups in relation to age, and the chi-square test was used in relation to gender. The Mann–Whitney *U* test was used to compare the nonglaucoma, glaucoma, and FAP groups in relation to aqueous humor EPO and serum EPO levels. The relation between EPO and serum was evaluated by Spearman correlation. Values of $p<0.05$ were considered statistically significant. Data analysis was performed using IBM SPSS Statistics software version 20.

RESULTS

A total of 21 glaucomatous eyes from 21 patients and 21 control eyes (21 patients) were enrolled in the study. The demographic characteristics of the patients are summarized in Table 1. Of the glaucoma eyes, ten were from FAP patients (group 1, mean age 55.4±10.0 years mean and standard deviation; five females) and 11 were from non-FAP patients (group 2, mean age 55.8±7.0 years mean and standard deviation; four females). Of the 21 control eyes, nine were from FAP patients with an indication for vitrectomy due to amyloid deposition (group 3, 55.9±8.5 years mean and standard deviation; four females) and 12 were from non-FAP patients awaiting phacoemulsification and intraocular lens implantation (group 4, 58.8±4.9 years mean and standard deviation; five females). Groups 1 and 2 presented indications for trabeculectomy,

Table 1. Demographic of the groups.

Age/Sex	Group 1 FAP glaucoma	Group 2 Non-FAP glaucoma	Group 3 FAP non-glaucoma	Group 4 Non-FAP non-glaucoma
Age (year, mean±SD)	55.4±10.0	55.8±7.0	55.9±8.5	58.8±4.9
Female/Male	5 / 5	4 / 7	4 / 5	5 / 7

Table 2. EPO in aqueous humor and serum of the groups.

Aqueous humor/ serum EPO level	Group 1 FAP glaucoma	Group 2 Non-FAP glaucoma	Group 3 FAP non-glaucoma	Group 4 Non-FAP non-glaucoma
Aqueous humor EPO level (mU/ml)	18.82±10.16	75.73±13.25	20.62±6.22	17.22±5.33
Serum EPO level (mU/ml)	13.44±4.82	9.99±2.84	15.04±5.87	8.73±4.12

Aqueous humor EPO (Mann–Whitney U test): Group 3 versus Group 4 p=0.23 ; Group 1 versus Group 2 p<0.001; Group 1 versus Group 3 p=0.29; Group 2 versus Group 4 p<0.001

had uncontrolled IOP (defined as IOP higher than the target pressure with maximally topical antiglaucoma medications: prostaglandin + beta blocker + anhydrase carbonic inhibitor + alpha-2 agonist), abnormal visual field test results, and abnormal cup/disc ratio.

The ages and gender distribution of the patients were similar between groups (Kruskal–Wallis test, p = 0.56; chi-square test, p = 0.94). All FAP patients had received an orthotopic liver transplant.

As summarized in Table 2, the mean EPO concentration in the aqueous humor of nonglaucomatous eyes (group 3 versus group 4) was not significantly different between FAP and non-FAP patients (20.62±6.22 mU/ml in group 3 and 17.22±5.33 mU/ml in group 4, p = 0.23) and corresponds presumably to the basal ocular production of EPO. In the presence of glaucoma, EPO concentrations in the aqueous humor showed a significant increase in the non-FAP group (group 2, 75.73±13.25 mU/ml; group 1, 18.82±10.16 mU/ml; p<0.001), and when we compared the non-FAP glaucoma group (group 2) with the nonglaucoma groups (FAP group 3 and non-FAP group 4), a similar finding was observed (p<0.001) (Table 2). In the FAP groups (group 1 and group 3), we observed no significant difference between the mean EPO values of patients with or without uncontrolled glaucoma (p = 0.29). As listed in Table 3, FAP patients with glaucoma (group 1) and non-FAP patients with glaucoma (group 2) were comparable in terms of the IOP (p = 0.39) and mean deviation (p = 0.75). The correlation between the IOP and the aqueous humor EPO was not significant in group 1 (mean IOP 26.20±1.93 mmHg; r_s = 0.02, p = 0.95) and group 2 (mean IOP 26.82±1.72 mmHg; r_s = 0.27, p = 0.41). There was also no significant correlation between the mean deviation and the aqueous humor EPO in group 1 (r_s = –0.48, p = 0.16) or group 2 (r_s = –0.07, p = 0.83).

Serum EPO levels among patient groups were not significantly different when multiple testing was taken into account (Bonferroni correction). No statistically significant correlation between the values of EPO in the serum and in the aqueous humor was observed in any patient (Spearman correlation coefficient r = 0.047, p = 0.77).

Table 3. Intraocular pressure and mean deviation in glaucoma groups.

IOP/mean deviation	Group 1 FAP Glaucoma	Group 2 Non FAP Glaucoma	P value Mann–Whitney test
N	10	11	
IOP, mmHg, mean±SD	26.20±1.93	26.82±1.72	0.39
Mean deviation, dB, mean±SD	−8.92±3.30	−8.26±3.63	0.75

DISCUSSION

Glaucoma is a manifestation of a heterogeneous group of diseases with a very complex and multifactorial pathophysiology [8]. Although hypotensive therapy is today the only possible therapeutic intervention, neuroprotective treatment strategies are emerging as a result of the advances in the comprehension of the pathophysiological mechanisms of glaucoma. In the future, neuroprotective agents will probably be part of the therapeutic arsenal available for the treatment of glaucoma. EPO has been shown to have a protective effect on ganglion cells against acute ischemia injury [28,47] and has been proposed as a potential neuroprotective treatment.

In this study we confirmed that the aqueous humor EPO level is higher in glaucomatous eyes than in nonglaucomatous eyes with cataracts, as previously reported [30-32,48,49]. This increase in aqueous humor EPO levels could be a result of local production and/or active transport through the blood–ocular barrier. This observation lends support to the hypothesis that EPO acts as an endogenous neuroprotector of retinal ganglion cells [19].

In spite of the inappropriately low renal EPO production reported in FAP ATTR V30M [44], its basal level in the aqueous humor of FAP patients was not significantly altered. However, FAP patients seemed to be unable to increase endocular EPO production in the presence of glaucoma. In previous studies, we showed an inappropriate secretion of renal EPO in FAP and an inability to increase EPO production in response to decreased serum hemoglobin levels, leading to a high incidence of anemia in these patients. The lack of response to glaucoma in FAP patients could be the ocular counterpart of the stunted renal EPO production in FAP in response to anemia.

It has been suggested that inhibition of EPO production could be caused by the toxicity of prefibrillar aggregates of TTR V30M [44,50,51]. These oligomers induce the expression of oxidative stress, pro-inflammatory cytokines, and apoptosis-related molecules [52,53] through the binding of TTR aggregates to the receptor for advanced glycation end products, activation of extracellular signal-regulated kinase cascades, and nuclear transcription factor kB [52-56], suppressing the EPO production. All our FAP patients had previously received an orthotopic liver transplant to eliminate their main source of mutant TTR, their own liver [57]. After liver transplantation, mutant TTR is removed from systemic circulation; however, its local production in the eye remains presumably unaffected. Therefore, the ocular pathology related to FAP, which includes glaucoma, continues to progress after liver transplantation; presumably there is also continuing deposition of cytotoxic prefibrillar TTR aggregates.

Garcia-Ramirez found that other factors besides hypoxia-inducible factors (HIF)-mediated hypoxia might be important in the upregulation of EPO. Hypoxia, ischemia, elevated reactive oxygen species, or increases in glutamate and nitric oxide caused by glaucomatous damage are probably the cause of elevated aqueous humor EPO concentration in chronic glaucoma [30]. The pro-inflammatory cytokines interleukin (IL)-1, IL-6, interferon-γ, and tumor necrosis factor (TNF)-α inhibit EPO production [58,59], but despite being increased in the aqueous humor of glaucoma eyes, as is especially the case for TNF-α [60], these cytokines do not prevent an increase in EPO levels.

Increased levels of TTR in the aqueous humor of glaucoma patients have been documented [61-63]. If glaucoma leads to an increase expression of TTR in the aqueous humor, an increased concentration of the unstable TTR V30M in FAP patients' eyes could contribute to the increased development of a mechanical barrier to the outflow of the aqueous humor [64], resulting in worsening the glaucoma. The association of open-angle glaucoma with autonomic nervous system dysfunction suggests that this could also play a role in the pathogenesis of the disease [65]. Patients with systemic sympathetic and parasympathetic neuropathies have a higher incidence of open-angle and normal-pressure glaucoma [66-69]. Because FAP patients have an early onset neuropathy with markedly autonomic involvement, it is likely that autonomic dysfunction plays a role in glaucoma pathophysiology. Other possible contributing factors are the hemodynamic instability often presented in FAP patients due to vascular deregulation and abnormal blood pressure that may compound the harmful effects of glaucoma, particularly during sleep [65].

In the groups with glaucoma, there was no correlation between the aqueous humor EPO concentration and the values of IOP and mean deviation. It seems that the concentration of EPO in the aqueous humor is not related to the IOP in eyes with glaucoma or previous eye injury caused by glaucoma.

In this study, patients with pseudoexfoliative and uveitic glaucomas were excluded because some studies pointed to blood–aqueous humor barrier breakdown in these situations [70,71]. EPO can cross the blood–brain barrier and blood–retina barrier [41]. We did not found a significant correlation between aqueous humor and serum EPO concentrations as other authors have found [30,31]. The elevation of the aqueous humor EPO level in glaucoma was not associated with a parallel increase in blood EPO levels, corroborating the role

of local EPO production as already proposed by Fu [28] and Garcia-Ramirez [29].

In conclusion, our study confirmed that the level of EPO is increased in aqueous humor of open-angle glaucomatous eyes, as found by other authors. This increase was not observed in FAP patients. With the increased survival of transplanted FAP patients, glaucoma prevalence is expected to increase dramatically with increased suvival of the transplanted patients. We showed lower endogenous neuroprotection in glaucomatous eyes of FAP patients, emphasizing the need for more aggressive glaucoma management to maintain vision through life.

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