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Contribution at the Study of Neuroprotective Properties of Neuroglobin during Severe Chronic Glaucoma

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Abstract

Introduction: The mechanisms of overexpression of neuroglobin in patients with severe glaucoma (CG^+) remain hypothetical. **Objective**: To study the anti-apoptotic, anti-hypoxic and anti-oxidant properties of neuroglobin in CG^+. **Population and Methods**: The visual field, as well as plasma dosage of neuroglobin (CmNgb, ng/ml), hypoxia inductible factor-1alpha (CmHIF-1*α*, pg/ml), glutathione peroxidase (CmGpx, pg/ml), and cytochrome C oxidase (CmCyt C, pg/ml) were carried out in 45 CG^+ and 45 controls (CG^-). The chi-2 test compared the proportions, and Spearman's test studied the correlations between quantitative variables ($p < 5\%$). **Results**: CmNgb was 4.1 in CG^+, *versus* 2.3 in CG^- ($p = 1.52 \times 10^{-5}$). CmGpx was 1144.7 in CG^+, *versus* 752.8 in GC^- ($p = 0.0199$). CmHIF-1*α* was 4.1 in CG^+, *versus* 3.5 in CG^- ($p = 0.4530$). CmCyt C was 2303.26 in CG^+, *versus* 1750.44 in CG^- ($p = 0.0450$). In CG^+, there was a correlation between CmNgb and CmGpx ($r = 0.417$; $p = 0.004$), CmNgb and CmHIF-1*α* ($r = 0.644$; $p = 1.8 \times 10^{-6}$), and between CmHIF-1*α* and CmGpx ($r = 0.447$; $p = 0.002$), CmHIF-1*α* and CmCyt C ($r = 0.371$; $p = 0.012$). None correlation was found between CmNgb and CmCyt C ($r = 0.126$; $p = 0.370$), as well as CmGpx and CmCyt C ($r = 0.102$; $p = 0.505$). **Conclusion**: The variations of apoptosis, hypoxic, and oxidative stress bio-

markers were found between CG^+ and CG^-, as well as their correlations, suggesting that neuroglobin overexpression is related to its anti-apoptotic, anti-oxidative, and anti-hypoxic properties.

Keywords

Chronic, Severe, Glaucoma, Neuroglobin, Neuroprotective-Properties

1. Introduction

Chronic glaucoma (CG) is an anterior, slowly progressive ischemic optic neuropathy [1] [2] [3] [4] [5]. It is characterized by a gradual loss of axonal fibers of the optic nerve, resulting from death of retinal ganglion cells (RGC) [6] [7] [8]. It is the second leading cause of blindness in the world [9] [10] [11]. Its pathophysiology is complex, and involves two theories. The first is mechanical, and has the major effect of compression of the head of the optic nerve by intra-eye hyper pressure. The second, is vascular, and involves hypoperfusion and ischemia-hypoxia of the optic nerve [12]-[18]. Despite these theories, only intra ocular pressure (IOP) remains the commonly modified risk factor during glaucoma. This situation makes that, all current pharmacological and surgical treatments aim to reduce IOP [19] [20] [21]. However, although this approach is effective for many patients with glaucoma, a significant proportion of their visual function deteriorates inexorably. In fact, about one in eight patients with glaucoma become blind in at least one eye despite this treatment [6] [21]. In animals, experimental therapies targeting and protecting retinal ganglion cells against phenomena related to hypoperfusion of the optic nerve (ischemia, hypoxia, oxidative stress, apoptosis) have been evoked and grouped under the term neuroprotection [6] [22]-[27]. Considered as neuroprotective molecule, neuroglobin (Ngb) is a protein belonging to the family of human globins [28]-[37]. It was discovered in 2000 by Burmester *et al.* [31]. It is a protein with preferential expression in the nervous system, with higher concentrations in the retinal and during optic nerve pathologies [28]-[35] [38]. Indeed, the work of Rajendram *et al.* had highlighted a tissue expression of neuroglobin correlated with the severity of glaucoma. This study was based on post-mortem retinal specimens from patients with a history of advanced chronic glaucoma [39]. *In vivo*, based on a plasma dosage in humans, Ovono et *al.*, in 2019, had noted an overexpression of plasma neuroglobin in patients with chronic glaucoma. This elevation depended not only on the severity, but also on the duration of the disease [40]. The authors suggested, moreover to the biomarker role of this globin, that of neuroprotective of the optic nerve during primary open-angle glaucoma, but without studying its mechanisms [39] [40]. We think that, during severe chronic glaucoma in human, the overexpression of neuroglobin observed *in vivo* is related to its neuroprotective properties, especially, anti-apoptotic, anti-hypoxic, anti-ischemic and

anti-oxidant. To confirm this hypothesis, we performed a plasma dosage of neuroglobin, biomarkers of hypoxia, oxidative stress, apoptosis, and analyze their correlations in patients with severe chronic glaucoma (CG^+).

2. Population and Methods

2.1. Population

This was prospective, cross-sectional and case control study, conducted from February 2018 to May 2019 in Libreville, Gabon. The recruitment of patients took place in the ophthalmology department of the University Hospital of Owendo. The conditioning and dosages of neuroglobin (Ngb), cytochrome C oxidase (Cyt C), glutathione peroxydase (Gpx) and inducible hypoxia factor-1 alpha (HIF-1α) were performed respectively in the biochemistry laboratory of the Faculty of Medicine Owendo, and the University Hospital of Angondjé. The study population of 90 people (180 eyes) had been divided into two groups. The first had 45 people (90 eyes) with bilateral severe chronic glaucoma, followed or newly discovered (patient, CG^+). The second had 45 healthy people (90 eyes), volunteers (controls, CG^-), with the same socio-demographic criteria as the patients.

2.2. Inclusion Criteria

After obtaining informed consent, all patients aged 15 to 70 years, with bilateral severe chronic glaucoma (interrogation, visual acuity, intra ocular pressure, pachymetry, vertical cup/disk ratio, visual field), and no recent history of pathology or ocular surgery were included. Moreover, people of the same age range as patients, without ocular pathology, had been recruited (interrogation, visual acuity, intra ocular pressure, pachymetry, vertical cup/disk ratio, visual field) [40].

2.3. Non-Inclusion Criteria

All persons having pathologies or taking drugs likely to modify the plasma concentration of neuroglobin [40] (diseases of the nervous system other than glaucoma, taking of deferoxamine, cobalt, cinnamic and valproic acid, iron chelator, 17β-œstradiol) and those suffering of others general diseases (diabetes, hypertension, neoplasia, chronic renal failure, auto immune disease) [28] [29] [30] [40] [41] had not been included. Moreover, people on treatment with anti-oxidant activity (conversion enzyme, hypolipidemic, anti-inflammatory, chemotherapy) were not included [40] [41] [42] [43] [44]. This investigation was conducted taking into account the principles of medical ethics according to the Helsinki declaration [45]. Indeed, it has been submitted for approval by the Gabon Ethics Committee (Authorization number 014/2018/PR/SG/CNE). In addition, the authorizations of the responsibilities in charge of the units in which the study had been carried out were obtained. Furthermore, volunteers and parents of patients were assured of the confidentiality of the data collected.

3. Methods

3.1. Recruitment of Study Population

-Subjects with severe glaucoma or CG^+

Patient recruitment was carried out consecutively in the ophthalmology consultation room of the Owendo University Hospital (CHUO). The examination began with an interrogation in which socio-demographic data, personal and family history, and lifestyle habits were collected. Then, an assessment of the central thickness of the cornea, visual acuity distance, intra ocular pressure (IOP), vertical cup/disk ratio (C/D) and visual field (VF) was carried out. All patients classified as severe glaucoma (CG^+) received treatment. Thus, all recent discovery CG^+ were put on treatment and then systematically reviewed four weeks later. On the other hand, previously diagnosed and regularly followed CG^+ did not change their initial treatment. Blood samples were taken during the second appointment in the recently discovered CG^+, and at the first appointment for CG^+ diagnosed before the study.

-Controls or CG^-

They were recruited voluntarily and consecutively within the general population. Participants were generally relatives of investigators or patients, medical staff and medical students. They also benefited from a blood sample, preceded by a clinical and functional evaluation carried out in the consultation room of the CHUO. This evaluation was the same that of patients (interrogation, intra ocular pressure, visual acuity distance, ratio cup/disk vertical and visual field).

During the study period, 150 people with eligibility criteria were identified, including 95 with severe glaucoma (CG^+), and 55 controls (CG^-). Of the 95 CG^+ recorded, 35 were not included due to high blood pressure, inflammatory pathologies (n = 10), diabetes (n = 5), smoking and alcoholic taking less than three weeks old (n = 20). Of the CG^+ included, 15 had been excluded due to refusal of blood test and unavailability. The CG^+ population definitively selected was 45 patients (90 eyes). Of the 55 selected controls, 45 (90 eyes) were included and nine (18 eyes) were not included for suspected glaucoma and a history of eye disease. One person was excluded from this group for refusal to take a sample. At the end of the survey, the CG^- selected population was 45 patients (90 eyes). A total of 90 people (180 eyes) participated in the study (**Figure 1**).

The variables studied were age (in years), gender, intra ocular pressure (IOP) in mmHg, vertical C/D ratio (decimal scale), mean deviation or MD (Decibel, -dB) [40], plasma concentrations of neuroglobin (ng/ml) [40] [41] [46] [47], glutathion peroxydase (pg/ml) [48] [49], cytochrome C oxidase (pg/ml) and HIF-1α (pg/ml) [49]. The plasma concentrations of Ngb, Gpx, cytochrome C and HIF-1α of controls (CG^-) were considered as reference values [40] [41] [46] [47] [48].

3.2. Diagnostic Methods and Classification of the Severity of Chronic Glaucoma

-Determination of intra ocular pressure (IOP)

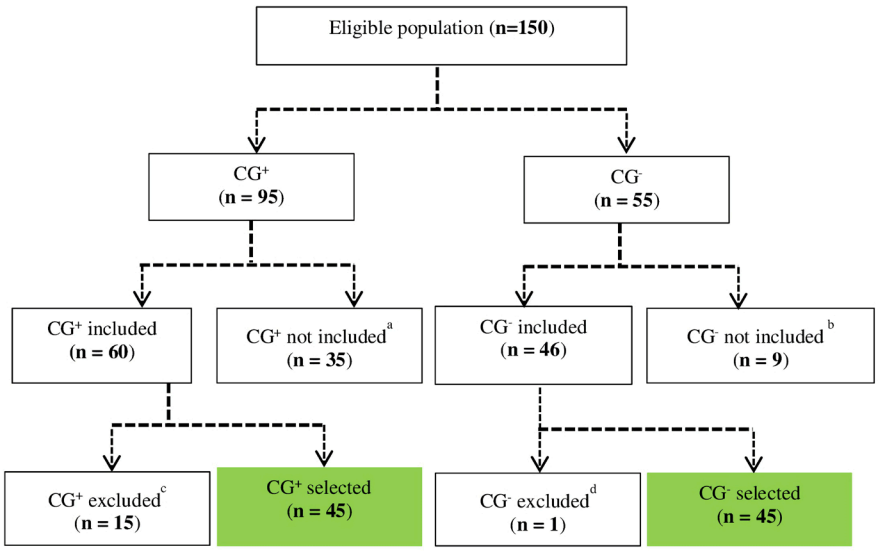

Figure 1. Flow diagram describing the selection of the study population. **Legend**: [a]Reasons of non inclusion in CG^+: high blood pressure, diabetes, alcoholism, smoking; [b]Reasons of non inclusion in CG^-: suspicion of glaucoma and history of eye disease; [c]Reasons of exclusion in CG^+: refusal to participate and unavailability; [d]Reasons of exclusion in CG^-: Unavailability and refusal for blood test.

In each participant, IOP measurement was performed using a Goldman applanation tonometer (TAT-100T®, TOMEY™, USA). The measurement procedure was carried out according to a protocol established during a previous study [40]. IOP was considered optimal for values below 21 mmHg [40] [50].

-Vertical Cup/disk ratio determination

The optical disk was clinically examined with the slit lamp (TSL-7000H®, TOMEY™, USA) using a Volk lens (SuperField NC®, Volk™, USA). The measurement procedure was carried out according to a protocol established by Ovono *et al.* study [40]. An excavation ≤ 0.3 was considered as normal [40] [49].

-Visual field evaluation method

The automated visual field (AP-3000®, TOMEY™, USA) was practiced in both glaucoma and controls. The procedure was carried out according to a protocol established during a previous study [40]. The visual field deficit was expressed in decibel (-dB). For this study, severe chronic glaucoma was defined by visual field with MD < −12 dB [40] [50] [51]. In the controls, a MD > −6 dB was considered as normal [40].

3.3. Biological Methods

-Blood collection and conditioning

Peripheral venous blood was taken after the installation of a tourniquet (COMED®, Clipcomed pro™, France). The equipment used for that was 10 ml syringes (NORM-JECT®, HSW™, Germany) and 26-Gauge catheter (Eurofine®, Euromedis™, France). Eight milliliters of blood were collected from two previously identified dry tubes and sent to the laboratory in a cooler (Coleman™,

USA). Upon arrival, the plasma were centrifuged (Hettich Zentrifugen®, Universal 320™, Germany), and the serum collected in cryovials (Biologix®, Biologix™, USA), then placed in a freezer (Thermo Scientific Forma 900 Series™, Thermo Fisher Scientific® Inc.) −80˚C.

-Plasma dosage of neuroglobine (Ngb)

The plasma dosage of neuroglobin was carried out by the technic ELISA (Enzyme linked immunosorbent assay). To do this, a Human NGB ELISA kit™ (N˚ E-EL-H1768) was used. The manual dosage procedure was conducted according to a protocol based on previous studies [40] [46] [47] [48]. The optical density was measured on a Biorad® PR 3100™ reader. The concentration of Ngb was obtained by comparison with a standard range treated at the same time as the samples. Plasma neuroglobin (CmNgb) concentrations were expressed in ng/ml [49].

-Plasma dosage of cytochrome C oxydase (Cyt C)

The cytochrome C oxidase assay was manually performed by the ELISA (Enzyme linked immunosorbent assay) technic. It was carried out using a Human cytochrome C oxidase ELISA kit™ (N˚ E-EL-H0056). The assay technic used in this study was previously described in the user manual provided by Elabscience® [49]. Plasma concentrations of cytochrome C oxidase (CmCyt C) were expressed in pg/ml [49].

-Plasma dosage of glutathione peroxidase (Gpx)

The plasma dosage of glutathione peroxydase (the dosage of the enzyme and not of activity) was carried out by the ELISA (Enzyme linked immunosorbent assay) technic, using a Human GPX1 ELISA kit™ (N˚ E-EL-H5410). The dosage procedure was done manually, following a protocol established in a previous study [46]. Plasma concentrations of Gpx (CmGpx) were expressed in pg/ml [46] [49].

-Plasma dosage of hypoxia-1 alpha inductible factor (HIF-1*α*)

Concerning HIF-1*α*, its plasma dosage was carried out by the ELISA (Enzyme linked immunosorbent assay) technic and using a kit Human HIF-1alpha ELISA kitTM (N˚ E-EL-H1277). The dosage procedure took place according to the manual dosage protocol proposed by Elabscience® [49]. Plasma concentrations of HIF-1*α* (CmHIF-1*α*) were expressed in pg/ml [49].

3.4. Statistical Methods

This investigation used a convenience sample, consisted of 90 people, including 45 patients with severe glaucoma (CG^+) and 45 controls (CG^-). The data, upon collected, was then entered on a Microsoft Excel 2010® file and analyzed using the EPI INFO 7® software of Center for diseases control, and SPSS® Statistic 21 for IBM®. The descriptive analysis was based on the calculation of means, medians, proportions and standard deviations. The comparison of proportions and means was based on the chi-square test. Spearman's test allowed us to study the correlations between quantitative variables. The relationships between quantitative and qualitative variables were analyzed by Pearson test ($p < 0.05$).

4. Results

4.1. Comparison of Epidemiological and Clinical Variables between Patients with Severe Glaucoma (CG^+) and Controls (CG^-)

Men accounted for 80% (n = 36/45) of CG^+, and 40% (n = 18/45) of CG^- ($p = 2.79 \times 10^{-5}$). Data from age comparison and clinical variables between CG^+ and CG^- are summarized in Table 1. The average age of the CG^+ was 51.30 ± 12.3 years and that of the CG^- 41 ± 9.5 years ($p = 2.79 \times 10^{-5}$). In CG^+, the average IOP to the right eye was 21.68 ±12 mmHg, compared to 12.2 ± 2.5 mmHg in CG^- ($p = 4.46 \times 10^{-6}$). The average IOP to the left eye, in CG^- was 21.55 ± 9.5 mmHg, compared to 12.23 ± 2.6 mmHg in CG^- ($p = 3.99 \times 10^{-8}$). The vertical cup/disc ratio (C/D) to the right eye was 0.96 ± 0.10 in CG^+, compared to 0.16 ± 0.11 in CG^- ($p = 0.1 \times 10^{-12}$). This ratio to the left eye was 0.98 ± 0.06 in CG^+, compared to 0.15 ± 0.10 in CG^- ($p = 0.1 \times 10^{-12}$). The mean MD to the right eye in CG^+ was −13.57 ± 1.55 dB and −3.45 ± 0.87 dB in CG^- ($p = 0.1 \times 10^{-12}$). The MD in the left eye was -14.5 ± 1.62 dB in CG^+, and −3.11 ± 0.76 dB in CG^- ($p = 0.1 \times 10^{-12}$).

4.2. Comparison of Biological Variables between Severe Glaucoma (CG^+) and Controls (CG^-)

The comparison of the average plasma concentration of neuroglobin (CmNgb) between CG^+ and CG^- shows that, in the CG^+, the CmNgb was 4.1 ± 2.3 ng/ml, *versus* 2.3 ± 1.2 ng/ml in the CG^- ($p = 1.52 \times 10^{-5}$) (Figure 2).

The data from the comparison of the average plasma concentration of glutathione peroxydase (CmGpx) between CG^+ and CG^- are illustrated in Figure 3. In the CG^+, the CmGpx was 1144.7 ± 737.7 pg/ml, compared to 752.8 ± 506.3 pg/ml in the CG^- (p = 0.0199).

The results obtained from the comparison of average plasma concentration of the inductible factor by hypoxia-1 alpha (CmHIF-1α) between CG^+ and CG^- were summarized in Figure 4. The CmHif-1α was 4.1 ± 2.9 pg/ml in the CG^+ and 3.5 ± 1.9 pg/ml in the CG^- (p = 0.4530).

Figure 5 represents the results obtained from the comparison of average plasma concentration of cytochrome C oxidase (CmCyt C) between CG^+ and GC^-. The CmCyt C was 2303.25 pg/ml in GC^+ *versus* 1750.43 pg/ml in GC^- (p = 0.0450).

4.3. Correlation between Biological Variables in Patients with Severe Glaucoma (CG^+)

The results from the analysis of the correlation between, the average plasma concentration of the inductible factor by hypoxia-1 alpha (CmHif-1α) and glutathione peroxydase (CmGpx) in CG^+ are illustrated in Figure 6. There was a positive correlation between CmHif-1α and CmGpx in CG^+ ($r = 0.447$; $p = 0.002$).

Table 1. Comparison of the averages (±SD) of epidemiological and clinical variables between CG^+ and CG^-.

Variables	CG^+	CG^-	p
Age (years)	51.3 (12.3)	41 (9.5)	2.79×10^{-5}
IOP[a] (mmHg)			
Right eye	21.68 (12)	12.2 (2.5)	4.46 × 10−6
Left eye	21.55 (9.9)	12.23 (2.6)	3.99 × 10−8
C/D[b] (décimal scale)			
Right eye	0.96 (0.1)	0.16 (0.1)	0.1×10^{-12}
Left eye	0.98 (0.06)	0.15 (0.09)	0.1×10^{-12}
MD[c] (−dB)			
Right eye	−13.6 (1.5)	−3.4 (0.86)	0.1×10^{-12}
Left eye	−14.5 (1.6)	−3.1 (0.76)	0.1×10^{-12}

[a]Intra ocular pressure; [b]cup/disk vertical ratio; [c]Mean deviation.

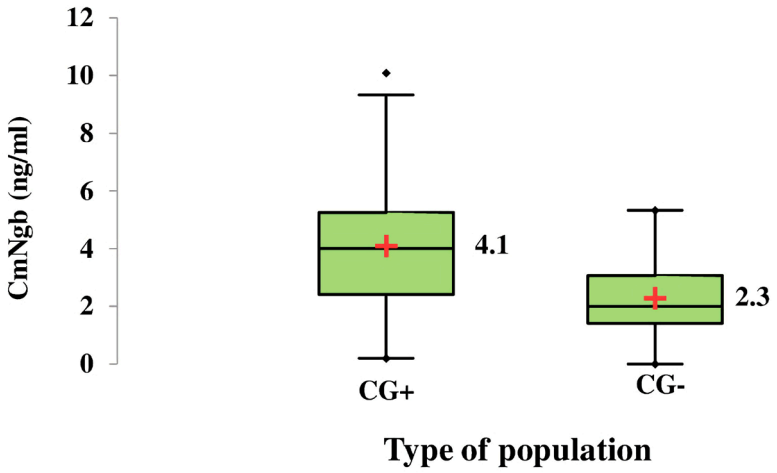

Figure 2. Comparison of CmNgb between CG^+ and CG^-.

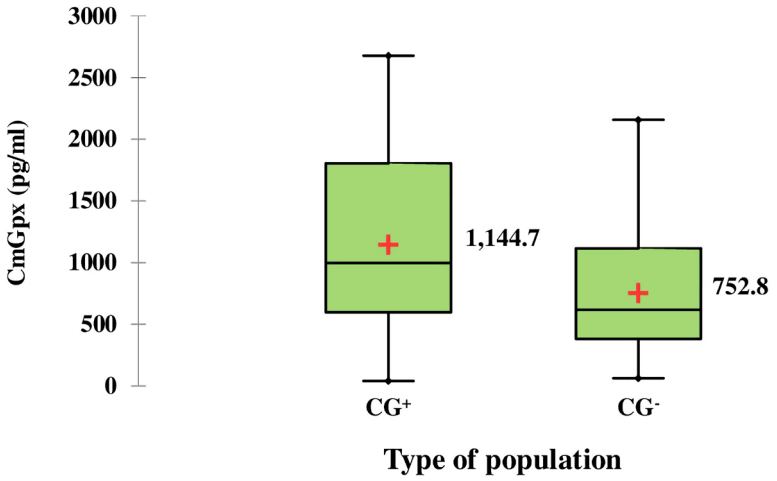

Figure 3. Comparison of CmGpx between CG^+ and CG^-.

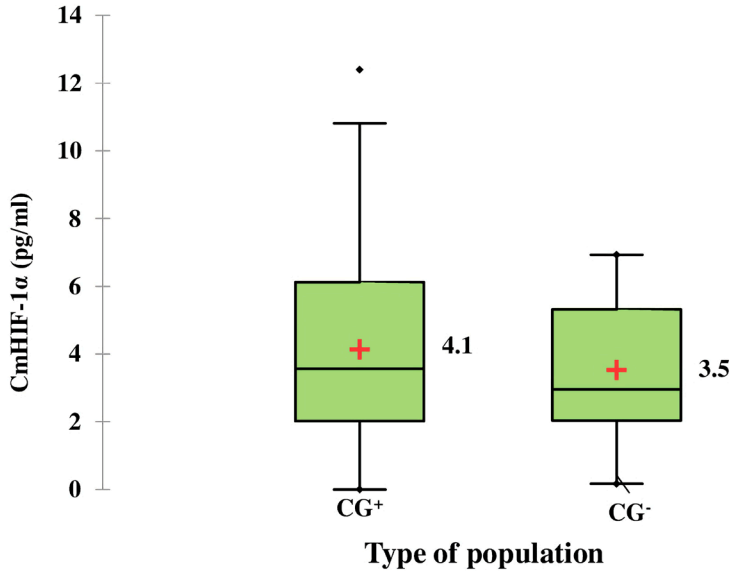

Figure 4. Comparison of CmHIF-1α between CG^+ and CG^-.

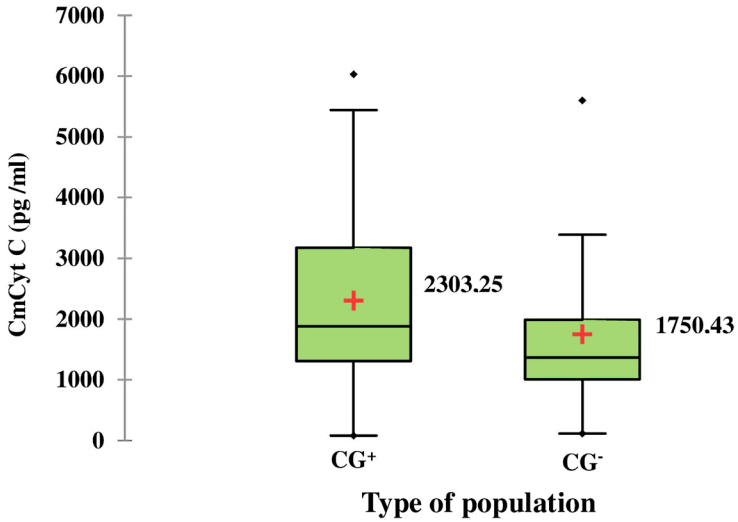

Figure 5. Comparison of CmCyt C between CG^+ and CG^-.

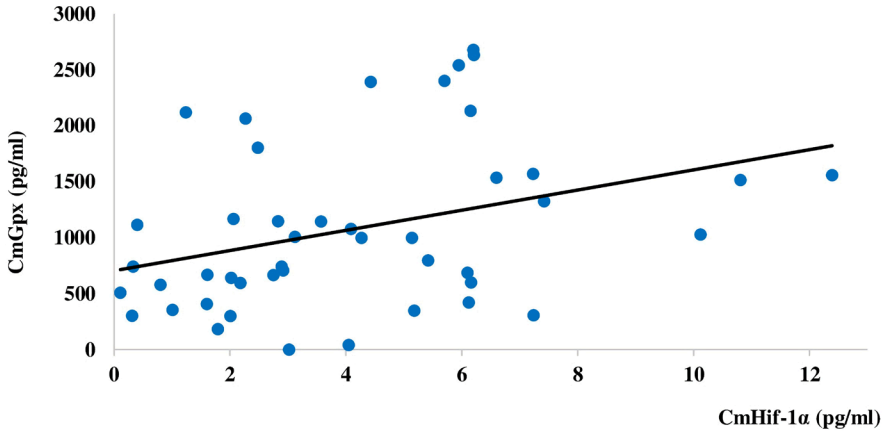

Figure 6. Correlation line between CmHif-1α and CmGpx in CG^+.

Data from the analysis of the correlation between average plasma neuroglobin concentration (CmNgb), and glutathione peroxydase (CmGpx) in CG^+ are illustrated in Figure 7. There was a positive correlation between CmNgb and CmGpx in CG^+ ($r = 0.417$; $p = 0.004$).

The results obtained from the analysis of the correlation between CmNgb and CmHif-1α in CG^+ are represented on Figure 8. There was a positive correlation between CmNgb and CmHif-1α in the CG^+ ($r = 0.644$; $p = 1.8 \times 10^{-6}$).

Data from the analysis of the correlation between CmHIF-1α and CmCyt C are represented on Figure 9. There was a positive correlation between CmHIF-1α and CmCyt C ($r = 0.371$; $p = 0.012$).

There was no correlation between CmNgb and CmCyt C ($r = 0.126$; $p = 0.370$), and between CmGpx and CmCyt C ($r = 0.102$; $p = 0.505$) (Figure 10, Figure 11).

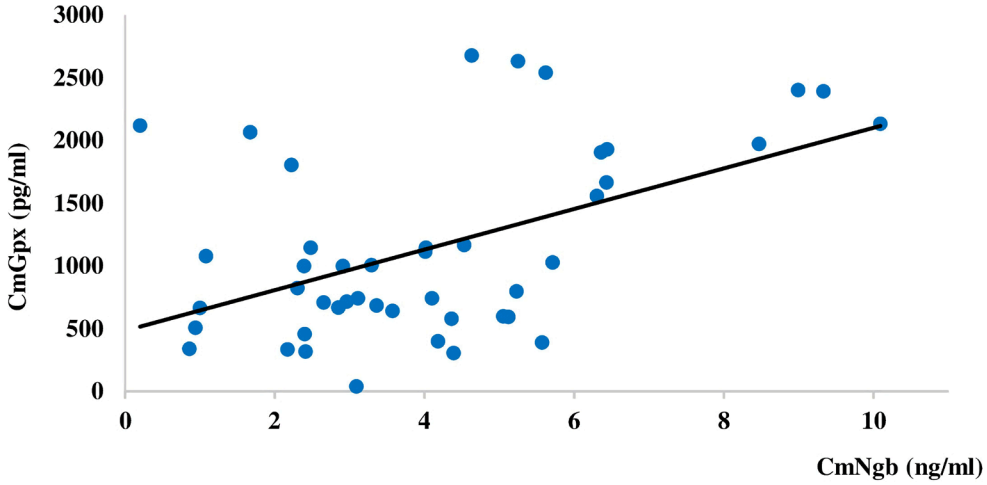

Figure 7. Correlation line between CmNgb and CmGpx in CG^+.

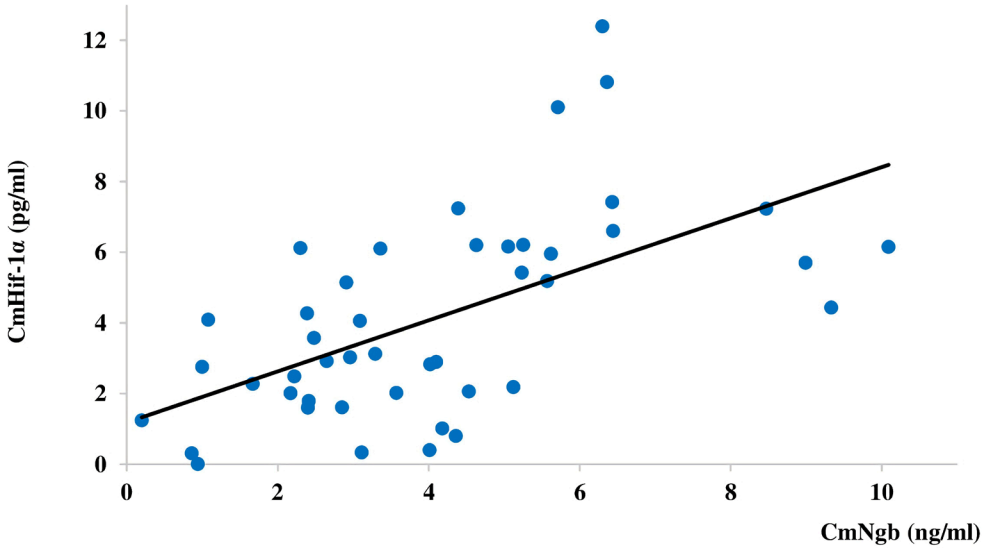

Figure 8. Correlation line between CmNgb and CmHIF-1α in CG^+.

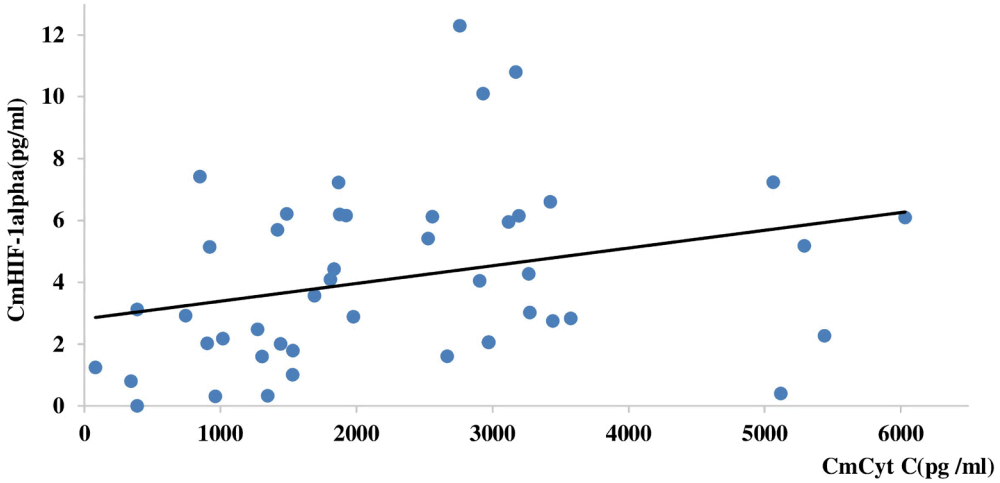

Figure 9. Correlation line between CmHIF-1alpha and CmCyt C in CG^{+}.

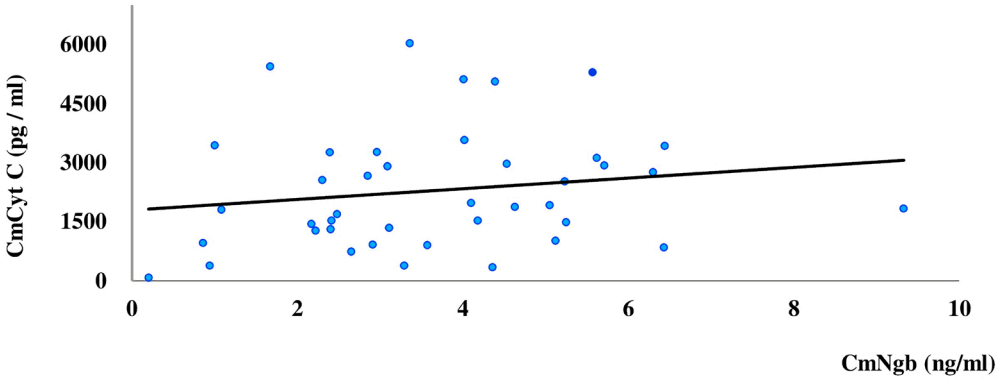

Figure 10. Correlation line between CmHIF-1alpha and CmCyt C in CG^{+}.

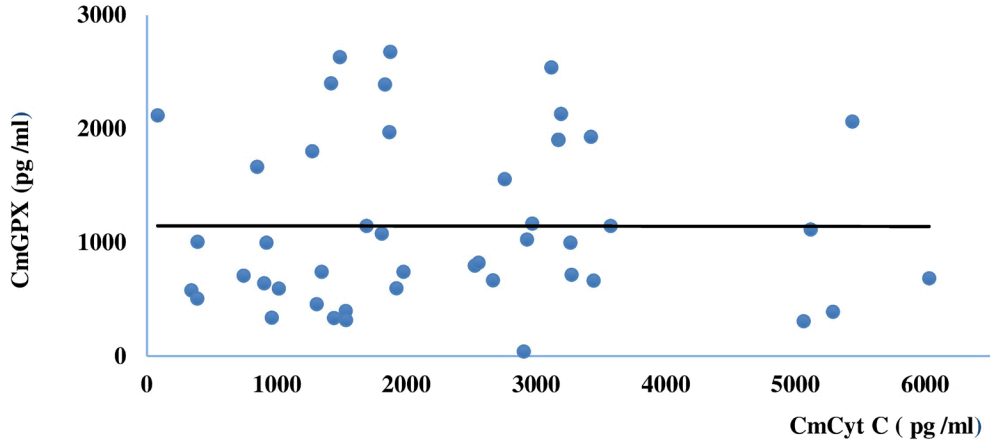

Figure 11. Correlation line between CmCyt C and Gpx in CG^{+}.

5. Discussion

5.1. Study Limits

Chronic glaucoma (CG) is an ischemic anterior optic neuropathy, resulting in blindness by progressive loss of axonal fibers of the optic nerve [1]-[8]. A study

conducted in human in 2019 found an overexpression of plasma neuroglobine that depended not only on the severity, but also of the evolution of chronic glaucoma, but did not explain its mechanisms [40]. We believe that during severe chronic glaucoma *in vivo* in human, observed neuroglobin overexpression is related to its neuroprotective properties, especially, anti-hypoxic, anti-ischemic anti-apoptotic and anti-oxidant. To confirm this hypothesis, we performed a plasma dosage of neuroglobin, biomarkers of hypoxia, apoptosis, oxidative stress, and analyzed their correlations in 45 patients with severe chronic glaucoma. During the recruitment phase of the participants, we meet several methodological difficulties. Above all, unavailability, refusal to participate and for blood test. All of these difficulties had the impact in sample size. However, despite these limitations, it appears that CmNgb was 4.1 ng/ml in CG^+, *versus* 2.3 ng/ml in CG^- ($p = 1.52 \times 10^{-5}$). The CmGpx was 1144.7 pg/ml in the CG^+, *versus* 752.8 pg/ml in CG^- ($p = 0.0199$). The CmHIF-1*α* was 4.1 pg/ml in GC^+, *versus* 3.5 pg/ml in CG^- ($p = 0.4530$). CmCyt C was 2303.26 pg/ml in CG^+, *versus* 1750.44 ng/ml in CG^- ($p = 0.0450$). In the CG^+, there was a correlation between cmNgb and CmGpx ($r = 0.417$; $p = 0.004$), CmNgb and CmHIF-1*α* ($r = 0.644$; $p = 1.8 \times 10^{-6}$), and between CmHIF-1*α* and CmGpx ($r = 0.447$; ($p = 0.002$), CmHIF-1*α* and CmCyt C ($r = 0.371$; $p = 0.012$). Conversely, there was no correlation between CmNgb and CmCyt C ($r = 0.126$; $p = 0.370$), as well as CmGpx and CmCyt C ($r = 0.102$; $p = 0.505$). So, plasma concentrations of markers of hypoxia, apoptosis, oxidative stress and neuroglobin are higher in CG^+ compared to CG^-. Furthermore, except from the cytochrome C oxydase, relationships were found between the different markers. In our humble opinion, these results suggest that during severe chronic glaucoma in humans *in vivo*, neuroglobin overexpression is related to its neuroprotective properties, especially, anti-ischemic, anti-apoptotic, anti-oxidant, and anti-hypoxic.

5.2. Comparison of Biological Variables in Patients with Severe Glaucoma (CG^+) and Controls (CG^-)

Comparison of CmNgb between CG^+ and CG^-

The CmNgb of the CG^+ was 1.8-fold higher than that of the CG^-. These results are similar to those of Rajendram *et al.*, and Ovono *et al.* [39] [40]. The fist authors had found higher levels of neuroglobin in *post mortem* collected retinal tissues in patients with advanced chronic glaucoma, compared to healthy people. This overexpression predominated in cells with high oxygen concentration *in situ*. These results suggested, according to them, an anti-ischemic role of neuroglobin due to its oxygen storage capacity during advanced glaucoma [39]. Ovono et *al.*, for their point of view, during a study conducted in human with chronic glaucoma, found plasmatic overexpression of neuroglobin correlated with the severity and duration of disease. Thus, glaucoma being a pathology in which hypoxia-ischemia processes are associated, the authors had suggested that neuroglobin had anti-ischemic properties, and had proposed it as a prognostic

biomarker [40]. Moreover, Nnang *et al.*, during a study with two types of populations, one of which having a stroke, and the other of healthy people (controls), had observed higher plasmatic levels of neuroglobin in patients with stroke compared to controls. According to Nnang *et al.*, these data suggested an anti-ischemic role of neuroglobine [48]. Therefore, our results confirm that during severe chronic glaucoma in human *in vivo*, there is an overexpression of neuroglobin, which is linked to hypoxia-ischemia phenomena [17] [39] [40] [52] [53] [54] [55].

On the other hand, the fact that the CmNgb of CG^+ is greater than that of CG^- allows us to discuss the intensity of expression of Ngb during severe chronic glaucoma (CG) compared to other situations of nervous system suffering (NS). Indeed, in the CG^+ population of this study, CmNgb was 8-fold higher than that found by Ovono *et al.* in a study of newborns suffering of anoxo-ischemic encephalopathy syndrome (4.09 ng/ml *versus* 0.50 ng/ml) [46].

Moreover, Nnang *et al.,* in a study of people with stroke had found plasmatic neuroglobine concentration 10-fold lower than our study (4.09 ng/ml *versus* 0.40 ng/ml). In that case, the results of our study support the idea that neuroglobin's expression increases not only in cases of suffering of NS in systematic way, but every more so during severe chronic glaucoma [28] [29] [30] [32] [33] [35]-[40]. Taking into account that neuroglobin overexpressed in severe chronic glaucoma *in vivo,* and that this elevation is function of the severity of glaucoma, our data suggest a neuroprotective role of this protein. That neuroprotection would be exercised *via* anti-ischemic, oxydant, hypoxic and apoptotic properties during severe chronic glaucoma.

-Comparison of CmGpx between CG^+ and CG^-

During our study, we found in CG^+, a CmGpx 1,5-fold higher to that of the CG^-. These results corroborate those of many other authors, including Goyal *et al.*, as well as Rokicki *et al.* [43] [55] [56] [57]. Indeed, the first authors, during in a study had determined the activity levels of glutathione peroxydase (Gpx) and non-enzymatic antioxidants in the aqueous humor of patients with primary open angle glaucoma, as well as in people with cataracts. They found an increase level of Gpx in the aqueous humor of patients compared to controls. The authors had suggested the presence of oxidative stress during glaucoma [56]. The second authors had evaluated oxidative damage, as well as variations of Gpx activity in red blood cells in patients with chronic glaucoma, and in people with cataracts. In their study, they observed an increase level of Gpx activity in the patients compared to those with cataracts [57]. According to them, these data also confirmed the presence of oxidative stress during glaucoma. Because glutathione peroxydase is considered a marker of oxidative stress [58]-[73], our results confirm the idea that, there is oxidative stress during severe chronic glaucoma [16] [17] [18] [56]-[61]. Otherwise, the elevation of Gpx observed could suggest it as a prognostic biomarker of oxidative stress during this pathology [56]. Furthermore, our results support the idea that, the administration of anti-

oxidants, additional to the conventional treatment of glaucoma would be beneficial for patients [7] [16] [22] [24] [61] [74].

Comparison of CmHIF-1*α* between CG^+ and CG^-

CmHIF-1*α* in CG^+ was higher compared to CG^- (4.1 ± 2.9 pg/ml in the CG^+ *versus* 3.5 ± 1.9) (p = 0.4530). These data corroborate those of other authors, such as Tezel *et al.* [75]. Indeed, they had studied hypoxic stress from a *post mortem* analysis of retinal tissue and heads of the optic nerves of glaucoma and control eyes. They found areas of retinal tissue and the head of the optic nerve with high concentrations of HIF-1*α*, exclusively in glaucoma subjects. Based on this finding, the authors had suggested the presence of hypoxic stress during glaucoma [75]. In the same order of ideas, Resec *et al.* had evaluated the expression of HIF-1*α* in the retina and head of the optic nerve of patients with advanced glaucoma. Always compared to the controls, the authors also found a great expression of HIF-1*α* within the same areas. Thereby, they concluded that hypoxia was present during glaucoma, probably mediated by HIF-1*α* [76]. Thus, our results confirm the presence of hypoxia during severe chronic glaucoma, as previously referred to by Childlow *et al.* [76] [77]. This hypoxia, according to Chen *et al.* [25], as well as Resec *et al.* [76], will be at the origin of the synthesis of HIF-1*α*. Thereafter induced HIF-1*α* would not only play a biomarker function, but also a neuroprotective role. However, the fact that this variation is not statistically significant would likely be related to the prolonged duration of hypoxia [61]. Indeed, it has been shown that HIF-1*α* is most often elevated only in acute, but non-chronic hypoxia, as is severe chronic glaucoma [12] [14] [17] [19] [60] [77]. An explanation for this result is related to the study population. In our sample, we have observed an important MD in CG^+. Indeed, in CG^+, the average MD was −13.6 dB in the right eye, and −14.5dB on the left (normal was -6dB during the present study) [40]. In the literature, an important MD involves an advanced glaucoma, *i.e.* with chronic hypoxia phenomena, but not acute, associated with apoptosis [17] [19], and the inhibition of induction of HIF-1*α* [25]. From this study, CmCyt C was 1.31-fold higher in CG^+, compared to CG^- (p = 0.0450). According to the other authors, apoptosis phenomena are one of the principal pathophysiological elements of chronic glaucoma [17] [77] [78] [79] [80] [81]. However, according to a recent study, cytochrome C oxidase is a highly sensitive biomarker of cellular apoptosis [82] [83]. Thus, an elevation of the plasma concentration of cytochrome C oxidase involves intense anti-apoptotic activity. Conversely, its decrease would be related to the decline of the one. A study of Daudt II *et al.* [84] on the efficacy of methylene blue in the degeneration of retinal cells ganglion in relation to apoptotic phenomena had noted a greater activity of cytochrome C oxidase in presence of this molecule. According to the authors, this suggested the efficacy of methylene blue against apoptosis of retinal cells ganglion *via* a likely activation of cytochrome C oxidase during chronic glaucoma. Thus, these data confirmed the hypotheses on relationship between cytochrome C oxidase and chronic glaucoma, already observed

by other contemporary authors [14] [15] [17]. We think that, the plasma elevation of cytochrome C oxidase observed in CG^+ confirms the presence of apoptosis phenomenon during severe chronic glaucoma in humans *in vivo* [14] [15] [17] [82]. Moreover, the relative difference of CmCyt C oxidase observed between the two groups could be related, either to the severity phenomena of glaucoma (cell necrosis, cell death), or because of the anti-apoptotic mechanisms triggered (for example, neuroglobin synthesis, cytochrome C oxidase itself, therapeutic factors).

5.3. Correlation of Biological Variables in Patient with Severe Glaucoma (CG^+)

Correlation of CmHif-1*α* and CmGpx in CG^+

On the one hand, during this work, a positive correlation between CmHIF-1*α* and CmGpx was found in the CG^+. On the other hand, we noted significantly higher concentrations of HIF-1*α* in CG^+ compared to CG^-. These two results could have several meanings. The first would be related to HIF-1*α*. In the literature, this protein is described as one of the sub-units of the protein complex HIF-1 actively involved in hypoxic pathologies. Indeed, according to Tezel *et al.*, as well as Resec *et al.*, it is considered a marker of hypoxia during glaucoma [75] [76] [77]. Thus, the variation of CmHIF-1*α* observed between CG^+ and CG^-, in our point of view, involves the presence of hypoxia-ischemia phenomena and the anti-hypoxic role played by HIF-1*α* during severe chronic glaucoma [75] [76] [77]. Another meaning would be related to glutathione peroxydase. This enzyme is known to be a marker of oxidative stress during reperfusion ischemia states [46], and ischemia hypoxia such as glaucoma [56] [57]. For it, according to the work of Goyal *et al.*, Rokicki *et al.*, as well as Marroco *et al.*, an increase in enzyme activity, and the plasma concentration of Gpx, involves oxidative stress during glaucoma [56] [57] [58]. Taking to account the data from Weinreb *et al.*, hypoxia is responsible of oxidative stress, that itself involves neuronal lesion, initially ischemic and then necrosis [17] [56] [57] [58] [59] [75] [76] [77] [82]. As a result, during our investigation, the variation of CmGpx found between CG^+ and CG^-, as well as the correlation between HIF-1 alpha and Gpx, suggest that during severe chronic glaucoma in human *in vivo*, hypoxia-ischemia phenomena are responsible for induction of oxidative stress.

Correlation between CmNgb and CmGpx in CG^+

A positive correlation was found between CmNgb and CmGpx during our study. These results could have several meanings. The first concerns the neuroglobin elevation in the plasma during chronic glaucoma. Indeed, it has been established that this protein is overexpressed in hypoxia-ischemia of the nervous system, and more specifically during chronic glaucoma [39] [40] [85] [86]. Otherwise, taking into account our data, a statistically significant difference in CmNgb was found between CG^+ and CG^- ($p = 0.004$). This result, in our opinion, suggests that Ngb overexpression observed is related to the hypox-

ia-ischemia phenomena already mentioned during glaucoma [17] [19] [25] [75] [76] [77] [87]. At the same time, it confirms the anti-ischemic role of this protein in relation to diseases of the nervous system [28] [29] [30] [32] [37] [42] [85], especially in chronic severe glaucoma in human [38] [39] [40] [52] [53] [54].

In parallel, another significance concerns glutathione peroxydase, which is considered by many authors as a biomarker of oxidative stress in general [58] [62]-[68] [70] [71] [73], and specifically during glaucoma [43] [56]-[61] [88] [89]. Anyway, during this survey, the CmGpx found in the CG^+ was 1.5-fold higher compared to CG^-. From this result, we have hypothetised the presence of oxidative stress in CG^+. So, according to many authors, during chronic glaucoma, the retinal cells ganglion undergo chronic hypoxia, which will generate oxidative stress [17] [88] [89]. This oxidative stress, thereafter would induce neuroglobin synthesis [28] [90]. Thus, during our work, the positive correlation found between CmNgb and CmGpx let's say us that in patients with severe chronic glaucoma, *in vivo*, oxidative stress is responsible for neuroglobin induction. As a result, our data suggest that this protein exerts anti-oxidant activity during severe chronic glaucoma. This anti-oxidant activity is likely responsible for the long-term preservation of the optic nerve during this disease [28] [29] [30] [31] [32] [38] [39] [52] [53] [54].

Correlation between CmNgb and CmHif-1*α* in CG^+

Regarding the relationship between CmNgb and CmHif-1*α*, a positive correlation was found in CG^+. This result supposes that an over-expression of Hif-1*α* is accompanied by an overexpression of Ngb and conversely. Furthermore, we found higher CmHIF-1*α* in CG^+ compared to CG^-. According to our opinion, this suggested the presence of hypoxia in CG^+. In the opinion of many authors, HIF-1*α* is considered as mediator of neuroglobin induction in the course of nervous system hypoxia conditions, such as severe chronic glaucoma [59] [75] [85] [86]. Moreover, according to Xie *et al.* [28], the overexpression of neuroglobin during pathologies of the nervous system such as glaucoma is related to its anti-hypoxic properties. For this reason, we think that the relationship highlighted between CmNgb and CmHif-1*α* during our study could mean that, in human, in the course of severe chronic glaucoma *in vivo*, there are hypoxia-ischemia phenomena. Thereafter, these phenomena are responsible for the overexpression of neuroglobin. Therefore, our results suggest an anti-hypoxic and anti-ischemic neuroprotective role of neuroglobin during severe chronic glaucoma in human [28]-[33] [36] [37] [38] [40].

Relationship between CmNgb, CmGpx and CmHif-1*α* in CG^+

During the present study, we found a positive correlation between and Gpx in CG^+. In literature, HIF-1*α* is considered like a biomarker of hypoxia, and Gpx that of oxidative stress [58] [59] [60] [75]. Accordingly, the relationship obtained demonstrates the presence of hypoxia and oxidative stress in the severe glaucoma in human *in vivo*. For this, taking into account the pathophysiology of glau-

coma, these results suggest that in CG^+, hypoxia-ischemia phenomena involve in oxidative stress activation [17] [77] [88] [89]. Similarly, a positive correlation was found between the Gpx and the Ngb. Because glutathione peroxidase is a biomarker of oxidative stress, and neuroglobin is overexpressed during ischemia-hypoxia situations of the optic nerve, we think that the positive correlation observed supposes that the induction of neuroglobin is related to oxidative stress in CG^+ [39] [40] [58] [60]. Regarding the precession of the induction of Ngb compared to that of oxidative stress, the result that we obtained shows that the overexpression of neuroglobin observed likely due to the oxidative stress phenomena. The existence of a positive correlation between CmNgb and CmHIF1-α allows us to also emit another hypothesis. Indeed, neuroglobin is considered as a biomarker of optic nerve ischemia during chronic glaucoma [39] [40] [47] [59]. Moreover, CmHIF-1α has been shown to be a biomarker of hypoxia during severe chronic glaucoma. Thus, these results mean that hypoxia found during severe chronic glaucoma is likely responsible for an elevation of Ngb, which would play its anti-hypoxic role [28] [29] [30] [31] [42] [78]. Based on these three hypotheses, we believe that during severe chronic glaucoma, there are phenomena of ischemia hypoxia and oxidative stress in the head of the optic nerve [59] [80]. These phenomena are thereafter responsible for neuroglobin overexpression [40]. This significant production of Ngb thus allows this molecule to fully play its neuroprotective role [12] [28] [29] [30] [31] [78] [79].

Relationship between CmNgb, CmCyt C and CmGpx in CG^+

In the CG^+, no correlation was found between CmCyt C and CmNgb on the one hand, but also between CmCyt C and CmGpx on the other. In contrast, CmCyt C, CmGpx and CmNgb were significantly higher in CG^+ compared to controls CG^-. The fact that plasmatic levels of cytochrome C oxidase are higher in CG^+ makes us suggest that there are apoptosis phenomena during severe chronic glaucoma. Indeed, cytochrome C oxidase being a plasma biomarker of anti-apoptotic activity during chronic glaucoma, its plasmatic activity and concentration decrease with the chronic course of apoptosis towards cell necrosis, or inversely towards its disappearance [14] [15] [17] [83]. In parallel, the plasmatic overexpression of neuroglobin observed in CG^+ *versus* controls made us suppose a neuroprotective role of this protein. This neuroprotection would be exercised through the anti-hypoxic, anti-ischemic, anti-oxidant and anti-apoptotic properties [28] [29] [30] [52] [53]. Glutathione peroxydase is considered as biomarker of oxidative stress [58]-[72]. The presence of higher plasmatic levels of this protein in CG^+ *versus* CG^- confirms the idea that there is oxidative stress during this pathology [16] [17] [18] [56]-[61]. Furthermore, this elevation could suggest glutathione peroxydase as a prognostic biomarker of oxidative stress during severe chronic glaucoma [56]. Thus, an explanation of the absence of correlation observed between CmCyt C and CmNgb but also between CmCyt C and CmGpx would be the fact that our sample consists mainly of severe glaucoma, probably in post apoptotic necrosis stage. Indeed, it has been shown that during chronic

glaucoma, the apoptosis, hypoxia and oxidative stress phenomena were correlated with the progression of the disease [17] [19] [78]. These would intensify to give way to the necrosis of the retinal cells ganglion, then *in fine,* those of the head of the optic nerve [12] [28] [29] [30] [31] [78] [79] [82] [83]. However, the presence of higher CmNgb and CmCyt C in CG^+ compared to CG^- made us accept the hypothesis that the absence of correlation found between CmCyt C and CmNgb is would be due to the anti-apoptotic activity of the Ngb. In other words, the significant synthesis of neuroglobin would have generated an anti-apoptotic effect which is responsible for a decrease of apoptosis process in the retinal cells ganglion, and those of the head of the optic nerve. These anti-apoptotic properties of Ngb have already been described by several other authors [29] [30] [38] [52] [53] [55]. Another hypothesis of this result would be the fact of the presence of a significant proportion of CG^+ in stabilization phase under the treatment. This stabilization would be reflected biologically by the decrease of cytochrome C oxidase plasma concentration. The same stabilization could also explain the absence of correlation between CmCyt C and CmGpx. Indeed, according many authors, the use of neuroprotective strategies would decelerate the suffering of retinal cells ganglion, and thus of oxidative stress and apoptosis [7] [12] [14] [16] [20] [22] [23] [24] [28] [29] [30] [31] [78] [79]. A cohort study including people with glaucoma and taking into account the variation of CmNgb according to the evolution of nerve damage under optimal therapy would be interesting. This would allow us to understand the role played by Ngb against vascular insufficiency (probably reponsible of hypoxia, then apoptosis and stress) during glaucoma, as these last lose their vision while they are on optimal treatment [82] [87]. In sum, our result corroborates the fact that during severe chronic glaucoma *in vivo* in humans, there are hypoxia-ischemia and apoptosis processes [12] [14] [15] [17] [25]. Those processes, at a certain duration of disease will be responsible for the synthesis of neuroglobin, *via* mediators such as HIF-1 alpha and cytochrome C oxidase. The chronically generated neuroglobin goes therefore plays an anti-apoptotic and anti-oxidant role. This chronic induction of Ngb would then influence the amplitude and progression of generating processes, and could be an explanation of the results obtained.

Correlation between CmHIF-1*α* and CmCyt C in CG^+

A positive correlation was found between CmCyt C oxidase and CmHIF-1*α* in CG^+. From this relationship, we can infer several meanings. According to recent studies, HIF-1*α* is a specific biomarker for hypoxic phenomena and has anti-hypoxic properties during disorders of the nervous system such as glaucoma [75] [76] [77]. Furthermore, during our work, it was noted plasma concentration of HIF-1*α* significantly higher in CG^+ compared to controls. In our opinion, the first meaning of this result would be the confirmation of the presence of hypoxia during severe chronic glaucoma, and suggests the neuroprotective role of HIF-1*α* in human *in vivo*. The second explanation of our result is said to be related to the confirmation of the existence of apoptosis phenomena in human,

and from this its neuroprotective role in patients with severe chronic glaucoma. Indeed, during our work, it was observed higher plasma levels of cytochrome C oxidase in CG^+. This data confirmed the existence of apoptosis phenomena during severe chronic glaucoma. This process, as described by many authors, will be responsible for the production of anti-apoptotic mediators such as cytochrome C oxidase [17] [80] [81] [82]. In fact, cytochrome C oxidase is a plasma biomarker of anti-apoptotic activity during chronic glaucoma. Thus, its plasma activity and concentration vary with the chronic evolution of apoptosis towards cellular necrosis, or conversely towards its disappearance [14] [15] [17] [83]. The positive correlation found between HIF-1 alpha and cytochrome C on the one hand, and on the other hand, the variations of concentrations observed within the two groups confirm the hypothesis that, during chronic glaucoma, hypoxia is responsible of anti-hypoxic mediators such as HIF-1*α*. Thereafter, this hypoxia is itself responsible for the activation of apoptosis phenomenon.

6. Conclusion

This work aimed to study the anti-apoptotic, anti-hypoxic and anti-oxidant properties of neuroglobin during severe chronic glaucoma in humans *in vivo*. To do this, we determined biomarkers of hypoxia, apoptosis and oxidative stress in people with severe chronic glaucoma (CG^+) and in controls (CG^-). At the end of our work, it appears that, compared to CG^-, plasma levels of Ngb, Gpx, cytochrome C oxidase and HIF-1alpha were higher in CG^+. The increased plasma concentrations of these molecules reflecting their anti-ischemic, anti-hypoxic, anti-oxidant and anti-apoptotic activities respectively, as well as the observed variations between the two groups suggest the presence of apoptosis, oxidative stress and ischemia hypoxia phenomena in CG^+. Otherwise, positive correlations were found in CG^+, between the plasma concentration of hypoxia and oxidative stress markers on the one hand, and between neuroglobin and these markers. Even if the precession of the steps remains to be demonstrated, our results suggest that in CG^+, by multiple complex processes which are being studied, hypoxia, ischemia, oxidative stress and apoptosis initially generated will induce probably in their turn, the synthesis of Ngb. The Ngb, *via* the amplification of the synthesis of the initial mediators, will fight against the processes at the source of the cascade. This situation, in our opinion, suggests the neuroprotective mechanisms of neuroglobin during severe chronic glaucoma. Thus, the various anti-ischemic, anti-apoptotic, anti-oxidant and anti-hypoxic mechanisms would generate the mitigation of generating processes, and could explain, at least in part, for example the absence of correlations found between apoptosis and other markers, or non-significant variation of HIF-1alpha between the two groups. Furthermore, we think that these overexpressed neuroprotective properties in the visual pathways are involved in the chronic evolution of the disease, reflecting not its natural evolution, but instead a help to the resistance capacity of retinal cells ganglion and head of the optic nerve.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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