Reproducibility of Optic Nerve Head Hemoglobin Measures

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Purpose: To evaluate intraobserver, interobserver, within-session and between-session reproducibility of the measurement of optic nerve head (ONH) hemoglobin levels by color analysis using Laguna ONhE [optic nerve hemoglobin (ONH Hb)] program.

Materials and Methods: This was an observational prospective study of 29 eyes (11 glaucomatous; 18 healthy eyes). Two examiners obtained 2 retinal photographs (Canon non-mydriatic retinal camera CD-DGi, Canon Inc.,Tokyo, Japan) in 2 testing sessions 3 weeks apart and analyzed the images using Laguna ONhE. The following parameters were quantified: ONH hemoglobin amounts across the whole disc (ONH Hb) and in the vertical disc diameter (8&20 Hb), cup-disc ratio (C/D), and the Glaucoma Discriminant Function (GDF). Agreement was illustrated using the Bland-Altman plots and reproducibility was assessed comparing the intraclass correlation coefficients (ICC).

Results: In session 1, examiner 1 found mean levels of ONH hemoglobin of $67.94 \pm 8.70\%$ in healthy eyes and of $57.90 \pm 5.36\%$ in glaucomatous eyes. Corresponding values for examiner 2 were $68.27 \pm 8.52\%$ and $57.83 \pm 4.88\%$, respectively. ONH Hb and 8&20 Hb measurements were lower in glaucomatous eyes ($P = 0.002$ and $P = 0.001$ respectively). GDF was also more pathologic in glaucomatous group. C/D ratio estimation was greater in the glaucoma group ($P = 0.003$). ONH Hb and 8&20 Hb showed the highest ICCs (all above 0.9). Variability was greater for GDF (ICC > 0.8) and C/D ratio estimation (ICC > 0.71).

Conclusions: Measurement of ONH Hb levels using the Laguna ONhE program shows high reproducibility both in glaucomatous and nonglaucomatous ONHs.

Key Words: optic nerve hemoglobin levels, optic nerve perfusion, glaucoma, retinal photograph

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The Laguna ONhE program was developed to measure hemoglobin (Hb) levels at the optic nerve Hb [ONH] by analyzing conventional fundus images.[1] The program is based on the finding that differences in optic nerve perfusion may be detected through changes produced in the color of the optic disc in retinal photographs. It is known that well-perfused tissues show greater oxygen saturation and Hb levels, and the important role played by optic nerve perfusion in eye diseases such as glaucoma is starting to emerge. In effect, this factor has been implicated both in the development and progression of glaucoma along with the known effects of elevated intraocular pressure on the optic disc.

Several studies have focused on determining oxygen levels at the disc level. Michelson and Scibor[2] detected reduced arteriole oxygen levels at the papilla in patients with normotensive glaucoma but not in those with primary open angle glaucoma (POAG) or ocular hypertension. Other authors[3] have observed that carbonic anhydrase inhibitors may increase oxygen saturation at the optic nerve head and propose that this increase is related to enhanced blood flow to the region.[4,5]

Some authors suggest the possibility that changes in optic nerve head reflectance could serve to detect differences in Hb levels.[6] Although the Hb amount of the optic nerve cannot be directly measured, optic nerve perfusion, as that of any tissue, depends on 3 factors: blood flow rate, Hb content, and oxygen saturation. Hence, by examining these factors, it is possible to identify zones in the disc containing lower or higher Hb levels. When there are a few different types of tissue in the region of interest and the intention is not to measure total perfusion but rather to determine oxygenation changes over time, it is useful to measure ≥ 2 wavelengths where oxy-Hb and deoxy-Hb show the same and different absorbance. The Laguna ONhE procedure was designed to identify Hb amount changes through the colorimetric analysis of the disc.[1,7] Within the papilla, artery zones show the highest levels of oxy- Hb and vein zones the lowest. On the basis of this biological reference, it is possible to determine differences in Hb levels across the different zones of the papilla. In effect, the disc cup features paler zones than the healthy neuroretinal rim. Thus, the main advantage of measuring oxy- Hb and deoxy- Hb in the papilla is that, based on a biological pattern of the patient him or herself (vein Hb amount), changes can be identified in the remaining papillary tissue. This determines that Laguna ONhE may be used as an objective tool to differentiate between normal and glaucomatous optic neuropathy in clinical practice. The technique is relatively easy to apply and inexpensive at the same time, as only a non-mydriatic retinal camera is needed, which is nowadays available in the majority of eye clinics. However, each camera requires a specific calibration of the function based on measurements acquired from a series of healthy subjects.

An essential quality in determining the utility of a device for clinical use practice is its measurement reproducibility. The goal of our present study was to determine the reproducibility of the measurement of ONH Hb levels

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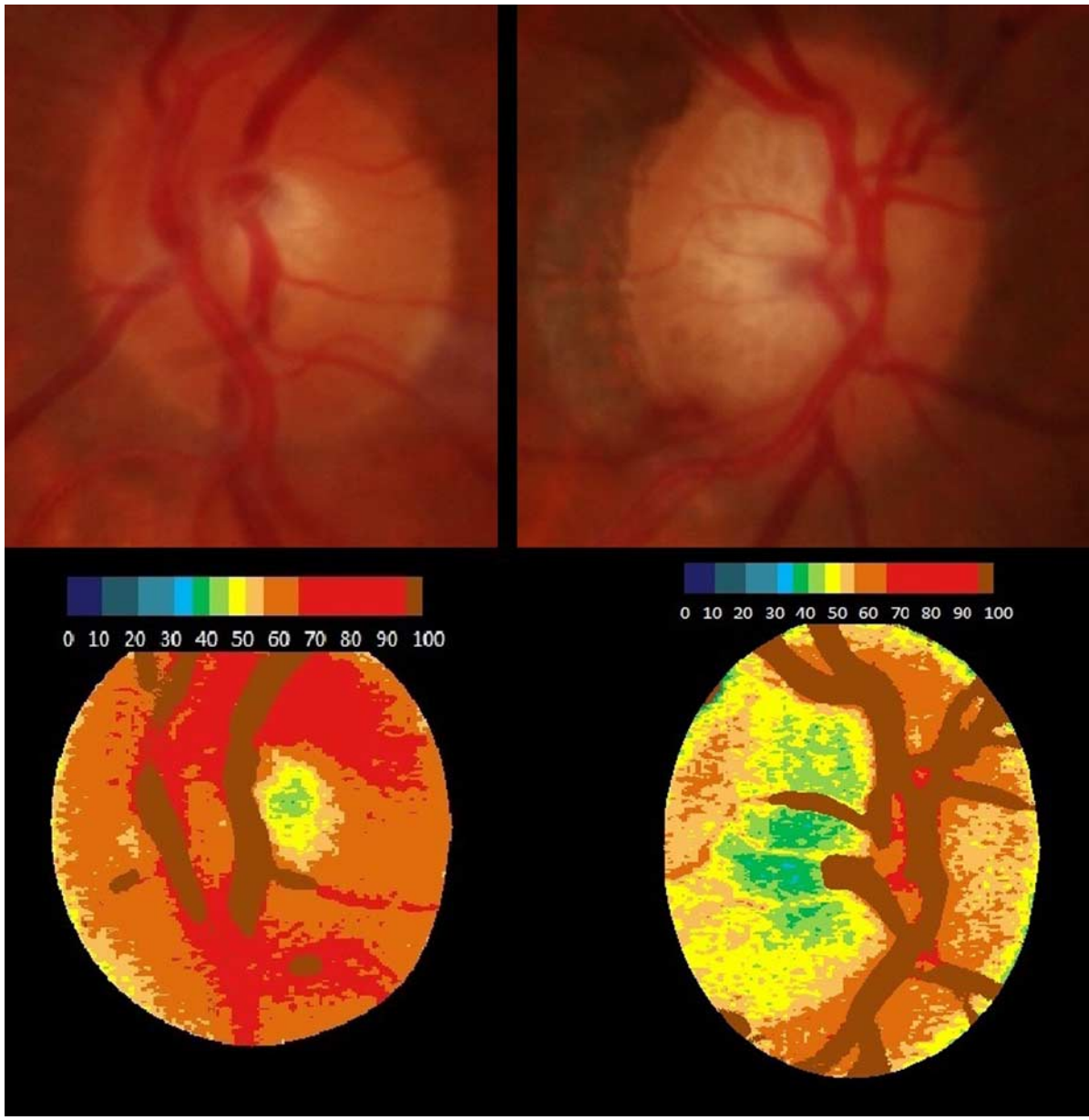

FIGURE 1. Examples of a normal (A) and a glaucomatous (B) papilla. Upper images are the color fundus photographs of the optic discs, whereas the lower images are their corresponding pseudo-images representing the amount of hemoglobin. A colorimetric scale is shown at the top of the lower images to assess the amount of hemoglobin. Figure 1 can be viewed in color online at www.glaucomajournal.com.

by color analysis using Laguna ONhE program in normal and glaucomatous human eyes.

MATERIALS AND METHODS

Subjects

The study protocol was approved by the local ethics committee and was conducted in accordance with the guidelines of the Declaration of Helsinki. Informed consent was obtained from each subject. A sample of 30 eyes of 30 subjects, 18 healthy eyes and 12 glaucomatous eyes referred for cataract surgery were selected consecutively and prospectively. Patients with glaucoma were enrolled from the glaucoma department of our hospital. Normal eyes were recruited from patients referred to our hospital for cataract surgery who underwent routine examination without abnormal ocular findings.

The inclusion criteria for both healthy and glaucomatous subjects were no history of ocular trauma or any other severe ocular disease. Individuals with diabetes, moderate or severe arterial hypertension (patients treated with ≥ 2 drugs), cardiovascular or hematologic diseases, as well as systemic diseases that could affect vision were excluded from the study. Additional inclusion criterion for the glaucoma group was a diagnosis of POAG.

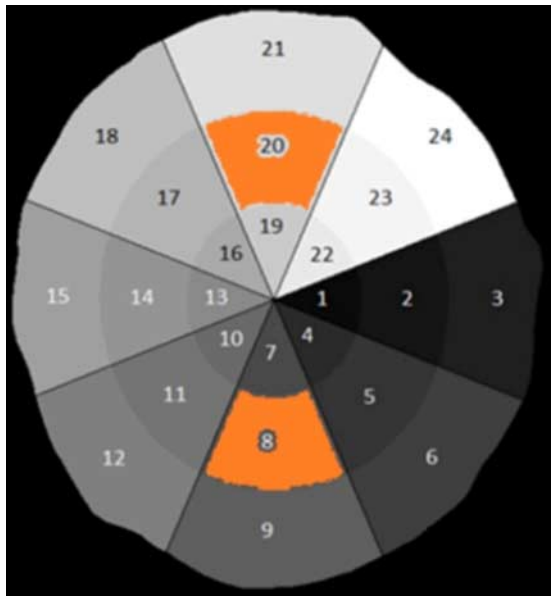

FIGURE 2. Image of a papilla automatically divided into eight 45-degree radial sectors and 3 concentric rings. In this study we have measured ONH hemoglobin amount in its whole extent and in vertical disc diameter, sectors 8 and 20 (8&20 Hb). The Laguna ONhE program uses an index called the Glaucoma Discriminant Function (GDF), which combines the slope of hemoglobin amount with the mean in sectors 8 and 20. Figure 2 can be viewed in color online at www.glaucomajournal.com.

Study Protocol

One eye from each subject was chosen randomly for the study. All subjects underwent an uncomplicated microincision cataract surgery (MICS) through a 1.8 mm incision, with implantation of a MICS IOL (CT ASPHINA 409 MP; Carl Zeiss Meditech AG, Goeschwitzer, Jena, Germany) by the same surgeon (C.M.-H.). Full blood test including hemogram and amount of Hb was performed weeks before surgery and always within a period shorter that a month before the surgery.

Full ophthalmic examination including refraction, best corrected visual acuity, intraocular pressure (Goldmann applanation tonometry), and slit lamp examination of the anterior and posterior segments was accomplished before surgery. All glaucoma patients had a POAG diagnosis that had needed previous medical or surgical treatment and had perimetric experience. All of them underwent visual field testing (G1-program of Octopus; Haag-Streit AG, Bern, Switzerland) before surgery having performed at least 2 previous reproducible examinations.

Two weeks after cataract surgery a new complete ophthalmic examination was performed including fundoscopy and retinal photographs to determine ONH Hb levels postoperatively.

TABLE 1. Demographic Characteristics, Amount of Hemoglobin Values in the Blood of Glaucoma Patients and Healthy Subjects, and MD Value for Glaucoma Patients

	Glaucoma	Controls	
	n = 18	n = 11	
	Mean (SD)		*P*
Age (y)	71 (11.16)	72.29 (8.22)	0.746
Female number (%)	9 (81.8)	14 (77.8)	0.794
Hemoglobin (mg/dL)	13.14 (1.78)	14.24 (1.10)	0.063
Mean Defect	5.78 (3.89)	—	—

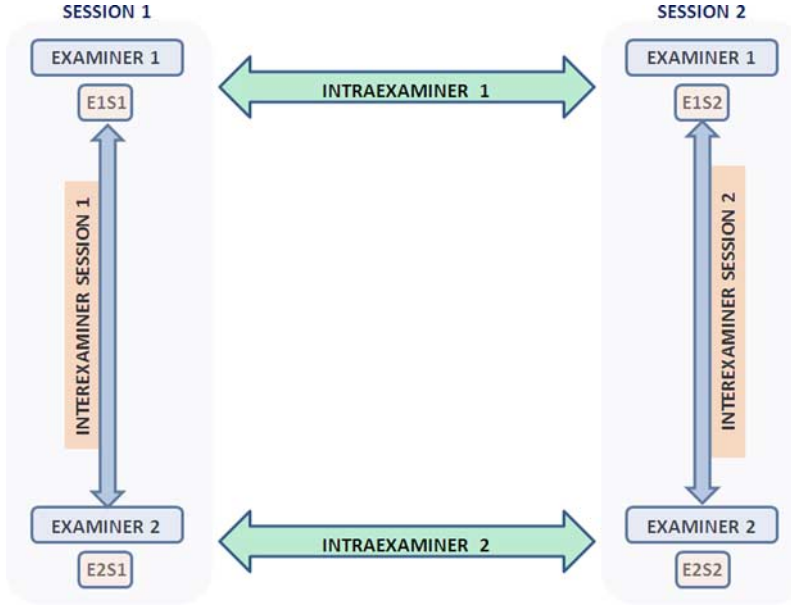

FIGURE 3. Study setup for the reproducibility of OnHE measurements. Patients were explored during 2 sessions by 2 independent examiners. Four retinographies (E1S1,E2S1, E2S2, and E2S2) were analyzed twice (analysis 1 and analysis 2) by an independent examiner in a masked manner. Figure 3 can be viewed in color online at www.glaucomajournal.com.

TABLE 2. ONH Hemoglobin Levels in its Whole Extent (ONH Hb) and in Vertical Disc Diameter (8&20 Hb), Cup-Disc Ratio (C/D) Estimation, and Glaucoma Discriminant Function (GDF) in Normal and Glaucomatous Eyes Determined in the First Testing Session by Examiner 1

	Mean (SD)		
	Glaucoma (n = 11)	Control (n = 18)	*P*
ONH Hb (%)	57.91 (5.63)	67.94 (8.70)	0.002
8&20 Hb (%)	61.54 (6.13)	72.72 (8.25)	0.001
GDF*	−1.00 (−6.00; 11.00)	26.50 (11.00; 37.25)	0.021
C/D	0.56 (0.09)	0.45 (0.09)	0.003

*Data was expressed as median (interquartile range).

8&20 Hb indicates ONH hemoglobin levels in its in vertical disc diameter; C/D, cup/disc ratio estimation; GDF, glaucoma discriminant function; ONH Hb, ONH hemoglobin levels in its whole extent.

ONH Measurements

All fundus photographs were taken using a Canon nonmydriatic retinal camera CD-DGi,(Canon Inc.,Tokyo, Japan). Laguna ONhE program analyzed 3 spectral components of ONH photographs: blue, green, and red.

The ONH areas with high Hb content mainly reflect red light. In contrast, areas with low Hb content reflect a lower proportion of the red component compared with the green and blue light. Figure 1 shows examples of a normal and a glaucomatous papilla with corresponding diagrams indicating Hb levels. Laguna ONhE program uses mathematical algorithms for automatic component segmentation to perform a semiautomatic delimitation of the ONH border and to identify the central retinal vessels. Two areas of the ONH were defined: the central retinal vessels and the ONH tissue itself. The formulas were then calculated at those pixels corresponding to the vessels as a whole and for every isolated pixel of tissue. Blue, green, and red components of the picture were assessed with an image analysis program using Matlab image processing toolbox (The MathWorks Inc., Natick, MA). The method whereby analyses Hb amount has been widely described in another article.[1]

Figure 2 shows how the Laguna ONhE program automatically divides the image of the papilla into 24 sectors shared across 3 concentric rings. The outer ring mostly aligns with the neuroretinal rim, the central ring corresponds to the transition area, which may include the neuroretinal rim and cupping, and the inner rim mainly comprises the cup area. On the basis of colorimetric analysis of the papilla, it is known that both in control individuals and patients with glaucoma, Hb levels are greater in the more peripheral sectors, that is, in the outer ring. The middle ring tends to show a lower amount of Hb than the outer ring and the innermost ring features the least amount of Hb. In other words, tissue Hb levels diminish from the disc periphery toward the center such that the difference between peripheral and central Hb levels can be calculated. By determining color changes in the central disc zone, the software estimates the cup and cup-disc ratio. It also uses an index called the Glaucoma Discriminant Function (GDF), which combines the Hb slope, obtained through multiple regression analysis of Hb estimates for each sector of the vertical meridian, with the mean Hb amount determined in sectors 8 and 20.

Two retinal photographs were taken by 2 independent examiners (examiners 1 and 2, corresponding to E1 and E2) in a randomized order who were masked to the identity of the patient in 2 testing sessions (session 1 and session 2, respectively), 2 weeks apart. Thus, 4 images were taken for both examiners E1S1, E1S2 for examiner 1, and E2S1 and E2S2 for examiner 2. Between each measurement, the subject was instructed to lean back before being repositioned on the headrest.

After the 2 sessions, an independent examiner analyzed the images twice (analysis 1 and 2, corresponding to A1 and A2, respectively) using Laguna ONhE in a masked manner (this independent examiner was unaware of the patient's diagnosis, nor who had graded the images, nor in which session these had been captured). Thus, 8 analyses were done for both examiners E1S1A1, E1S1A2, E1S2A1, and E2S2A2 for examiner 1 and E2S1A1, E2S2A1, E2S1A2, and E2S2A2 for examiner 2. Interobserver, intraobserver, and interanalysis reproducibility were calculated. Figure 3 shows the scheme of the experimental setup.

Statistical Analysis

All statistical analyses were performed using the IBM SPSS (version 17.0; IBM Corp., Somers, NY) statistical software.

The following parameters were quantified: ONH Hemoglobin amount in its whole extent and in vertical disc diameter (sectors 8 and 20), cup-disc ratio estimation, and

TABLE 3. Intraclass Correlation Coefficients (ICC) Along With the Corresponding 95% Limits of Agreement for the ONH Hb Amount in its Whole Extent (ONH Hb) and in Vertical Disc Diameter (8&20 Hb), Cup-disc Ratio (C/D) Estimation, and Glaucoma Discriminant Function (GDF) Obtained in Analysis 1 and 2

	8&20 HB	C/D Ratio	ONH HB	GDF
Analysis 1				
Intraexaminer 1	0.963 (0.921-0.983)	0.836 (0.654-0.923)	0.980 (0.958-0.991)	0.904 (0.796-0.955)
Intraexaminer 2	0.962 (0.910-0.983)	0.841 (0.665-0.925)	0.975 (0,947-0,988)	0.906 (0.799-0.956)
Interexaminer session 1	0.946 (0.886-0.975)	0.894 (0.773-0.950)	0,963 (0.922-0.983)	0.912 (0.811-0.959)
Interexaminer session 2	0.978 (0.954-0.990)	0.935 (0.863-0.969)	0.986 (0.969-0.993)	0.955 (0.905-0.979)
Analysis 2				
Intraexaminer 1	0.965 (0.926-0.984)	0.930 (0.852-0.967)	0.975 (0.948-0.988)	0.947 (0.886-0.975)
Intraexaminer 2	0.960 (0.902-0.982)	0.924 (0.833-0.965)	0.966 (0,926-0,984)	0.942 (0.867-0.973)
Interexaminer session 1	0.952 (0.897-0.977)	0.909 (0.806-0.957)	0.958 (0.911-0.980)	0.922 (0.833-0.963)
Interexaminer session 2	0.972 (0.939-0.987)	0.932 (0.858-0.968)	0.983 (0.963-0.992)	0.951 (0.897-0.977)
Interanalysis	0.987 (0.973-0.994)	0.930 (0.852-0.967)	0.992 (0.984-0.996)	0.962 (0.919-0.982)

*Intraclass Correlation Coefficient (95% confidence interval)

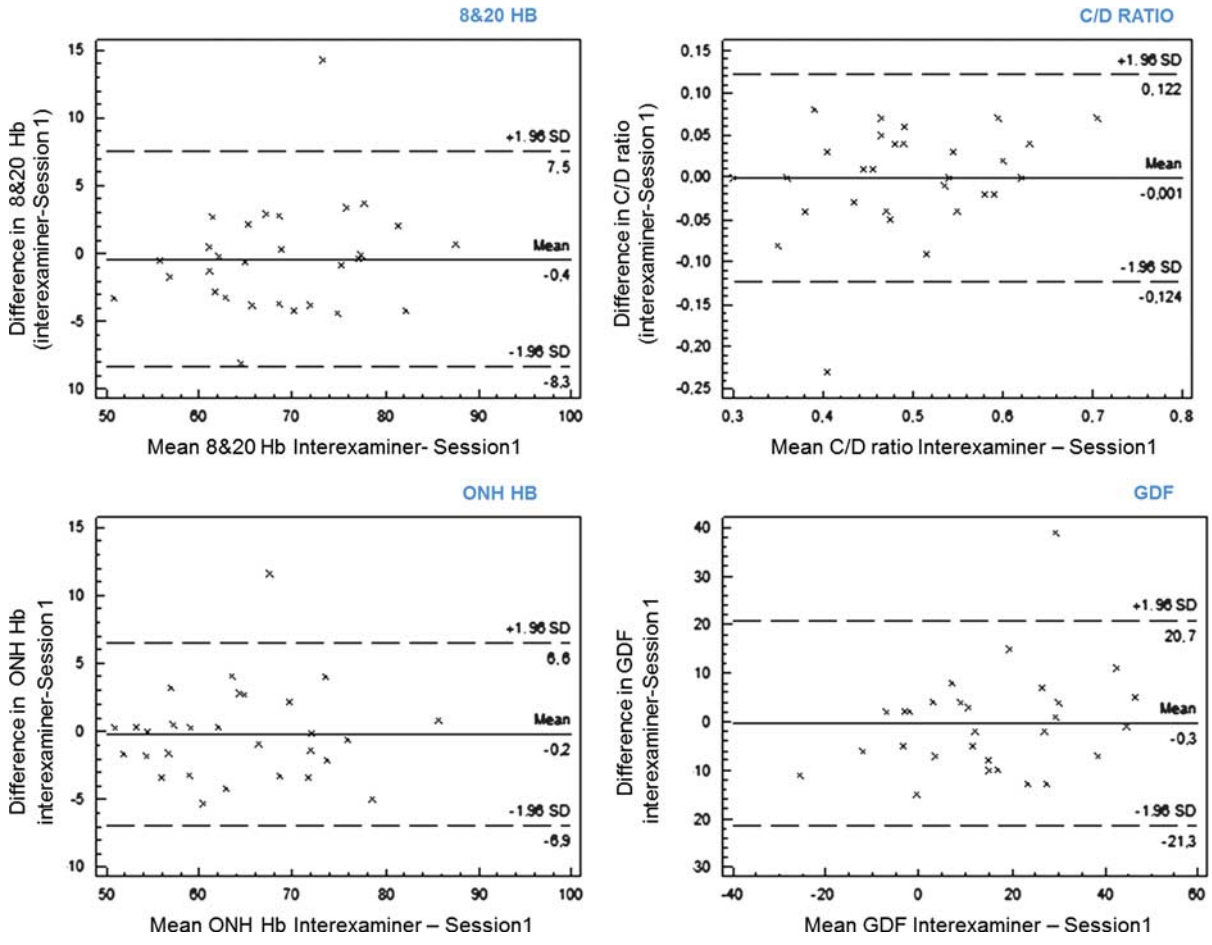

FIGURE 4. Differences in ONH Hb amount in its whole extent and in vertical disc diameter (8&20 sector), cup-disc ratio estimation, and GDF, measured in the first session, between the 2 examiners plot against their average, with the mean difference and 95% limits of agreement depicted by the 3 lines. Figure 4 can be viewed in color online at www.glaucomajournal.com.

GDF, which combines the slope of Hb amount with the mean in sectors 8 and 20 (Fig. 2). Descriptive statistics for quantitative variables, such as means and SD, as well as relative frequencies for qualitative variables such as sex, were conducted. A 2-sample *t* test was used to determine whether significant differences existed in the ONH Hb amount in its whole extent, Hb amount in vertical disc diameter and cup- disc ratio estimation and GDF between healthy and glaucomatous eyes.

The reproducibility of Laguna OnHE was assessed by comparison of the test-retest repeatability.[8]

Test-retest repeatability was determined for all parameters studied, comparing measurements obtained with Laguna OnHE by the same examiner in the first and second session.

Agreement was illustrated using the Bland-Altman plots. Reproducibility was assessed comparing the intraclass correlation coefficients (ICC) along with the corresponding 95% limits of agreement.

RESULTS

Thirty eyes of 30 subjects, 18 healthy eyes and 12 glaucomatous eyes were examined. One patient in the glaucoma group had to be excluded because of poor quality of the fundus photographs after cataract surgery that made Laguna OnHE analysis impossible to be performed and measure Hb levels.

Twenty-nine retinal photographs of 29 patients, 18 healthy eyes and 11 glaucomatous eyes were included.

Table 1 shows the characteristics and demographics, amount of Hb values in the blood of the study sample, as well as MD value for glaucoma patients. There were no significant differences in sex (0.794), age distribution (0.764), or Hb values in the blood (0.063) between glaucoma patients and healthy controls.

Table 2 shows ONH Hb levels in its whole extent and in vertical disc diameter, cup-disc ratio estimation, and GDF in normal and glaucomatous eyes determined in the first testing session by examiner 1. ONH Hb levels in the glaucoma group were significantly decreased compared with healthy controls [mean decrease of −10.03, 95% confidence interval (CI): −16.09, −3.98 in Hb levels in its whole extent and mean decrease of −11.17, 95% CI: −17.09, −5.26 in 8&20 sectors], and cup-disc ratio estimation was greater in glaucoma group (mean difference 0.11, 95% CI, 0.04-0.19).

Intraclass correlation coefficients (ICCs) are represented in Table 3. All ICC values obtained were above 0.8,

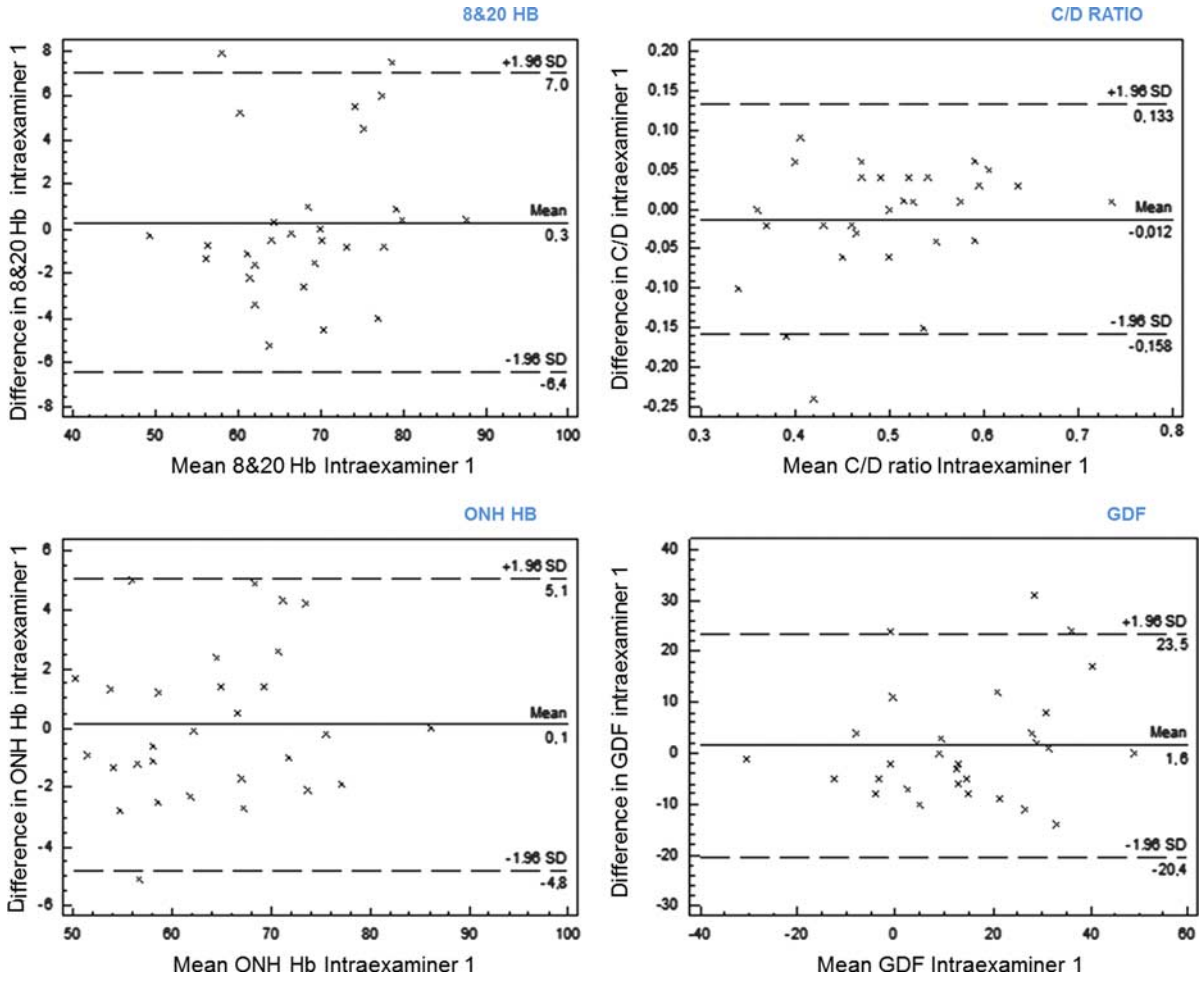

FIGURE 5. Differences in ONH Hb amount in its whole extent and in vertical disc diameter (8&20 sector), cup-disc ratio estimation, and GDF, measured in the 2 sessions by the examiner 1 plot against their average. Figure 5 can be viewed in color online at www.glaucomajournal.com.

with the mean ONH Hb amount in its whole extent measurement having the highest ICC, and the cup-disc ratio estimation measurement having the lowest. Figure 4 shows a the Bland-Altman plot for differences in mean ONH Hb amount in its whole extent and in vertical disc diameter (8&20 sector), cup-disc ratio estimation, and GDF values for the interobserver reproducibility in session 1 along with the corresponding 95% limits of agreement. Figure 5 shows a Bland-Altman plot for differences in mean ONH Hb amount in its whole extent and in vertical disc diameter (8&20 sector), cup-disc ratio estimation, and GDF values for the intra-examiner 1 reproducibility along with the corresponding 95% limits of agreement.

Reproducibility of none of the parameters studied was affected by magnitude of measure.

DISCUSSION

The ONH color depends on the amount of Hb it contains. Using conventional retinal photographs it is possible to measure the amount of Hb of the ONH.[1,7]

In areas with high Hb content, most of the light reflected is red, less is green, and even less is blue. Arteries reflect more red, much less green, and even less blue. Veins reflect less red than arteries, and very little blue and green at similar amounts, because the venous blood is less oxygenated. Tissue regions, which have good perfusion, such as the neuroretinal rim, reflect more green and blue than the central retinal vessels, because they have less Hb. In poorly vascularized tissue (eg, cupping) the proportion of reflected green and blue increases, and this is perceived in the image as whitening.

Laguna ONhE program obtains a reproducible measurement of Hb amount in ONH because of the reference pattern, which is the Hb level at the central retinal vessels on their way through the ONH, which are affected by the same variables. Thus, Hb may be measured at each point or sector of the ONH using the same formula to define the chromatic characteristics of the tissue and the reference vessels.[1,9]

The loss of lens transparency due to cataracts produces diffusion of light in fundus images. This produces an increase in the green component of the vessels because of light coming from the tissue. After cataract surgery, the green value decreases and the blue value increases as a consequence of the reduced diffusion. The differences

between red and green and between red and blue decrease and, therefore, the area appears whiter. The effects of lens deterioration on vessels and on tissue are proportional, when measuring the Hb levels at the vessels, the extent of the diffusion effect on the tissue may be estimated. In this way, it is possible to compensate its effect on the Hb estimation. Despite this, and with the objective of avoiding artefacts due to cataract, all patients included in this study were pseudophakic and had the same intraocular lens.

Optic nerve perfusion depends on Hb content, blood flow rate, and oxygen saturation. To avoid bias patients with cardiovascular factors such as hypercholesterolemia, diabetes, cardiovascular, or hematological disorders were not included. Therefore, ONH perfusion levels have not been affected by those factors. Besides, all patients included presented blood Hb levels within normal; hence, this factor has not affected ONH Hb levels. Retinal photography was performed in a period shorter than a month as blood test was done; therefore, it can be considered that values presented no changes during this period.

We also know that high variability of normal human optic disc morphology such as different disc sizes, as well as refractive errors, may affect measurement accuracy as well as other imaging technologies used in glaucoma diagnosis. To avoid the influence of these factors patients with refractive errors higher than 5 Dp spherical equivalent or 3 Dp astigmatism, as well as oblique or tilted ONH were excluded.

In our study, all the ICC values obtained were above 0.8 and only 3 of them presented values under 0.9. Even the lower 95% CIs were >0.65, indicating excellent reproducibility of all measurements. ICCs were all over excellent with the mean ONH Hb amount in its whole extent measurement having the highest ICC (above 0.95 in all the analysis performed) and the cup-disc ratio estimation having the lowest, but still excellent, (ICCs over 0.80 in each case). Moreover, the Bland-Altman plots showed no significant bias toward greater or lesser values in all the parameters analyzed.

A recently published study shows its high reproducibility, good diagnostic capability, and diagnostic agreement with other morphologic procedures as well as a good correlation with most of the morphologic and functional indexes of POAG.[1] Laguna ONhE program has also showed good reproducibility measuring Hb percentages in multiple sclerosis patients and healthy subjects.[7]

Tissue perfusion depends on various factors, such as blood volume, blood velocity, and the degree of oxygenation. Changes in oxygen saturation of the ONH have been measured previously[2–5,10–13] and several studies have measured blood volume using reflectometry,[6,14,15] but these authors have not described reproducible methods to measure the amount of Hb. According to the results obtained in our study, Laguna ONhE has an excellent reproducibility interobserver and intraobserver for all the analyzed parameters in pseudophakic patients with no cardiovascular risk factors associated.

Besides, results showed a high reproducibility with the 2 analysis performed by another masked examiner. The parameter which has shown lower reproducibility was cup-disc ratio estimation. Laguna ONhE program displays a cup-disc ratio based on Hb amount estimations in the vertical axis, although it's main objective is not to measure the cup-disc ratio. The precise value of this variable will depend both on papillary color changes and morphologic features such as the cup slope, which are more difficult to measure using Laguna ONhE compared with other structural glaucoma diagnosis methods. Despite this limitation, reproducibility values for these parameters were also high. Laguna ONhE program is relatively easy and not expensive to apply because only a fundus camera is needed.

In conclusion, measurement of ONH Hb amount using Laguna ONhE program shows high reproducibility both in glaucomatous and nonglaucomatous pseudophakic patients so that it could be considered an useful alternative in structural glaucoma diagnosis.

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