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Estimating the Amount of Hemoglobin in the Neuroretinal Rim Using Color Images and OCT

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ABSTRACT

Purpose: To calculate the amount of hemoglobin (Hb) in the optic nerve head (ONH), using superimposed color fundus images with disc, rim and cup boundaries obtained by OCT-Cirrus.

Material and Methods: We examined 100 healthy and 121 glaucomatous eyes using Oculus–Spark perimetry, Cirrus-OCT and Visucam (Zeiss) ONH color images. The Laguna ONhE program was then used to calculate the amount of Hb in the cup and six sectors of the rim. Receiver operating characteristic (ROC) analysis was performed and correlations between parameters were calculated.

Results: In suspected and confirmed glaucoma, Hb was significantly lower than controls in all rim sectors, especially the inferior and superonasal (p < 0.0001). Mean deviation (MD) of visual field regions showed greater correlation with the amount of Hb in the superior and inferior sectors of the rim than with rim area (p = 0.02) or nerve fiber layer thickness (p < 0.0001). On ROC analysis, the best diagnostic indicators were OCT rim area, vertical cup/disc ratio (C/D) and Glaucoma Discriminant Function (GDF) of Laguna ONhE, without significant differences.

Conclusions: The amount of Hb in the ONH seems to have an important relationship with glaucomatous visual field sensitivity. The remaining rim has insufficient perfusion in many cases of glaucoma.

Keywords: Glaucoma, hemoglobin, optic nerve head, perfusion, perimetry, visual field

INTRODUCTION

In recent years, numerous methods of undoubted merit have emerged for the examination of glaucoma, but many of them often lead to frustration in clinical practice. Their elevated cost hinders universal application for a disease with such high prevalence. It is therefore important to assess the cost/benefit ratio of diagnostic and monitoring methods. Just as the simplification of perimetry has facilitated practical application, shortening examination time, simplifying the instruments required and, ultimately, reducing costs, we believe it is important to seek other efficient methods to improve diagnosis that are accessible to most users.

The three main components of perfusion of the optic nerve head (ONH) are: first of all, blood velocity, which may be indirectly estimated at neighboring arteries by Doppler or locally with complex methods of laser doppler1 or laser speckle flowgraphy.2 Second, blood oxygen saturation; initial interest in this technique for the study of glaucoma3 has decreased, due to instrumental difficulties and contradictory results.4,5 Third, the amount of circulating blood, which had not been previously quantified, and that was the objective of our work using the Laguna optic
nerve hemoglobin (ONhE) method published in
2013. Also, a recent publication has introduced a
technique to measure ONH vascularization using
OCT angiography.

Our 2013 publication resulted in an interesting
suggestion by Dr Denniss to analyze the relative
amount of hemoglobin present in the rim and in the
cup, separately. In our response, we discussed two
possible alternatives: one would be direct application
of the Laguna ONhE method to color images of the
ONH obtained by stereophotography with three-
dimensional reconstruction of the shape of the
nerve. The other would involve superimposing Hb
maps with disc and cup boundaries obtained by OCT.
We applied the former method in a recent work. The
latter approach was used in this study.

MATERIALS AND METHODS

This study was performed in accordance with the
ethical standards laid down in Declaration of Helsinki
(World Medical Association, WMA, 64th General
Assembly, Fortaleza, Brazil, October 2013), and was
approved by the Institutional Review Board of the
University Hospital of the Canary Islands. The
participants were informed about the study objectives
and signed informed consent was obtained from all
of them.

A sample of 100 healthy eyes and 121 glaucomat-
ous eyes were consecutively and prospectively
selected. Normal eyes were recruited from patients
referred for refraction undergoing routine examina-
tion without abnormal ocular findings, hospital staff
and relatives of patients in our hospital. Patients with
glaucoma were enrolled from the Department of
Ophthalmology, University Hospital of the Canary
Islands, Tenerife, Spain. One eye from each subject
was randomly chosen for the study, except when only
one eye met the inclusion criteria.

Eligible subjects had to have a best-corrected visual
acuity of 20/40 or better, refractive error within ±5.00
diopters equivalent sphere, and ±2 diopters astig-
matism, and an open anterior chamber angle. The
presence of cataract was not considered a criterion
for exclusion a priori. Age, previous cataract or
glaucoma surgery were not criteria for exclusion.
Patients with any other associated eye disease that
could produce alterations in the optic disc and
interfere with the interpretation of the results were
excluded from the study.

Study Protocol

Participants underwent a full ophthalmologic exami-
nation: clinical history, visual acuity, biomicroscopy
of the anterior segment using a slit-lamp, intraocular
pressure (IOP) measurement and ophthalmoscopy of
the posterior segment.

All glaucoma patients had perimetric assessment,
having undergone at least two previous examinations.
The white-on-white Spark strategy was used in an
Easyfield perimeter (Oculus Optikgeräte GmbH,
Wetzlar, Germany). An abnormal perimetric
result was defined as a reproducible glaucomatous
visual field loss in the absence of any other abnor-
malities to explain the defect.

Photographs of the optic disc were obtained
using a Visucam non-mydriatic fundus retinograph
(Carl Zeiss Meditec). The peripapillary retinal nerve
fiber layer (RNFL) thickness and ONH parameters
(rim area, disc area, cup volume and vertical C/D
to ratio) were measured using the optic disc cube
200 x 200 acquisition protocol (software version 5.2)
of the Cirrus spectral-domain optical coherence
tomograph (OCT; Carl Zeiss Meditec Inc., Dublin,
CA). Left eye data were converted to a right eye
format. All images were acquired with a quality
greater than 6/10.

The images obtained with OCT with their disk, rim
and cup boundaries were adjusted in size and
orientation to fit ONH color images, using a computer
program we have designed specifically for this pur-
pose. After superimposing the two images, the
Laguna ONhE program was used to calculate the
relative amount of Hb in the cup and the six sectors of
the rim into which it is usually divided. In addition,
the program analyzed 24 sectors of the ONH, as
described in our first study, divided evenly using 8
radiuses and two rings located at one and two thirds
of the radius.

The Laguna ONhE program uses mathematical
algorithms for automatic component segmentation to
identify the central retinal vessels. Thus, two areas of
the ONH were defined: the central retinal vessels and
the ONH tissue itself. The program analyzed three
spectral components of ONH photographs: centered
on blue (B), green (G) and red (R), but not monochro-
matic, and applied the formula (R-G)/R to the pixels
of vessels and tissue. The result obtained for the
vessels was used as the reference value for calculating
the Hb content in the tissue. The (R-G)/R value was
calculated for any area of the tissue, then divided by
the (R-G)/R value for the vessels and the result was
multiplied by 100. Finally, the influence of the lens
status was compensated for by analyzing the differ-
ences between the green and blue components before
calculating the results of the Hb amount. The blue,
green and red components were assessed with
an image analysis program using Matlab image
processing toolbox (The MathWorks Inc, Natick,
MA) (Figure 1).

The program provides an index called Glaucoma
Discriminant Function (GDF), which combines the
results of relative Hb obtained in various sectors of
FIGURE 1 Photograph of a glaucomatous optic nerve head (upper left). Delimitation of the same optic nerve head using OCT (upper right). Superimposition of the two images (lower left). Hemoglobin map and sectorial results (lower right).

TABLE 1 Demographic and clinical characteristics of both study groups.

<table>
<thead>
<tr>
<th></th>
<th>Control group (Mean ± SD)</th>
<th>Glaucoma group (Mean ± SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.1 ± 14.5</td>
<td>64.1 ± 13.1</td>
<td>0.14*</td>
</tr>
<tr>
<td>Rim area (OCT)</td>
<td>1.38 ± 0.26</td>
<td>0.84 ± 0.33</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>C/D (OCT)</td>
<td>0.45 ± 0.16</td>
<td>0.71 ± 0.15</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Average RNFLT (OCT)</td>
<td>92.7 ± 9.8</td>
<td>72.2 ± 18.9</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>GDF (Laguna ONhE)</td>
<td>67.7 ± 43.2</td>
<td>−37.6 ± 73.2</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>MD of Spark perimetry (dB)</td>
<td>0.3 ± 1.8</td>
<td>−6.4 ± 9.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>PSD of Spark perimetry (dB)</td>
<td>1.5 ± 0.5</td>
<td>3.9 ± 3.0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>36/64</td>
<td>63/58</td>
<td>&lt;0.05**</td>
</tr>
<tr>
<td>N</td>
<td>100</td>
<td>121</td>
<td></td>
</tr>
</tbody>
</table>

SD = Standard deviation; C/D = vertical cup-to-disc ratio; RNFLT = retinal nerve fiber layer thickness; GDF = Glaucoma Discriminant Function; MD = mean deviation; PSD = pattern standard deviation; M/F = male/female; N = number of cases. p < 0.05 was considered statistically significant (in bold).

*Student t-test.

**Chi-square test.
the optic nerve to differentiate between glaucoma and normality.6 All the ophthalmic examinations, perimetry tests and morphologic evaluations were performed within 1 month of the subject’s date of enrollment in the study.

Classification into Groups

Healthy eyes had an IOP of less than 21 mmHg, no history of increased IOP, normal optic disc morphology and normal visual field results. The glaucoma group comprised subjects with POAG, pseudoexfoliative glaucoma and pigmentary glaucoma. Glaucomatous eyes had focal (localized notching) or diffuse neuroretinal rim narrowing with concentric enlargement of the optic cup, or both, and/or abnormal perimetry, regardless of the IOP values. In addition, we included a group of cases with IOP > 30 mm Hg, or IOP > 21 mmHg plus family history of glaucoma or suspicious looking optic disc or visual field, but with MD > –3 dB, considered cases of suspected glaucoma.

Statistical Analysis

All statistical analyses were performed using the Statistica software version 6.0 (StatSoft Inc., Tulsa, OK) and MedCalc version 7.3 (MedCalc software, Mariakerke, Belgium). The areas under the receiver operating characteristic curves (AUCs) were calculated for all parameters of every test. Sensitivities at a fixed specificity around 95% (5% false positive rate) were compared between the parameters with the largest AUCs.

Using the amounts of Hb in the 24 sectors into which the ONH was divided in the initial work, we estimated vertical C/D ratio and rim area by means of stepwise multiple correlation. After checking for a normal distribution of the variables, Pearson correlations between the structural and functional parameters were also calculated.

RESULTS

Only three cases were excluded: two very advanced cataracts and one intense miosis which prevented the capture of images. Finally, we analyzed 100 normal eyes and 121 eyes in the glaucoma group, of which 49 were of glaucoma suspects. Mean deviation (MD) ± standard deviation of Spark perimetry was 0.3 ± 1.8 dB in healthy subjects and −6.4 ± 9.6 dB in the glaucoma group (Table 1). We did not observe any variations in the amount of Hb in relation to age in normal subjects ($r^2 = 0.0025$, $p = 0.61$).

Glaucomatous eyes presented significantly less amount of Hb than normal eyes for the cup and all sectors of the rim ($p < 0.05$). The difference was minimal in the temporal sector ($p = 0.049$) and greatest in the inferonasal and superonasal sectors ($p < 0.0001$; Table 2).

For the whole sample, the best correlation between instruments using linear regression was between the Laguna ONhE total relative Hb amount and the OCT vertical C/D ratio (Table 3 and Figure 2). In glaucoma patients, we compared relative Hb values, rim area and thickness of the fiber layer, corresponding to the two superior and two inferior sectors of the ONH, with MD values in the sectors corresponding to the visual field.15 A higher correlation of sectorial MD with the relative amount of Hb

<table>
<thead>
<tr>
<th>Sector</th>
<th>Control group</th>
<th>Glaucoma group</th>
<th>$p^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb cup</td>
<td>56.2</td>
<td>44.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hb rim (121–230°)</td>
<td>76.7</td>
<td>69.1</td>
<td>0.0007</td>
</tr>
<tr>
<td>Hb rim (231–270°)</td>
<td>76.5</td>
<td>66.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Hb rim (271–310°)</td>
<td>67.2</td>
<td>55.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hb rim (311–40°)</td>
<td>51.8</td>
<td>46.9</td>
<td>0.049</td>
</tr>
<tr>
<td>Hb rim (41–80°)</td>
<td>70.5</td>
<td>59.7</td>
<td>0.0002</td>
</tr>
<tr>
<td>Hb rim (81–120°)</td>
<td>84.0</td>
<td>72.8</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Hb = Amount of hemoglobin in the sector (%). A $p$ value of <0.05 was considered statistically significant (in bold).

*Student $t$-test.

<table>
<thead>
<tr>
<th>TABLE 3 Coefficients of determination ($r^2$) between the different indices; $p &lt; 0.01$ for all comparisons.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>GDF</td>
</tr>
<tr>
<td>OCT rim area</td>
</tr>
<tr>
<td>OCT C/D</td>
</tr>
<tr>
<td>OCT RNFLT</td>
</tr>
<tr>
<td>VF MD</td>
</tr>
<tr>
<td>VF PSD</td>
</tr>
</tbody>
</table>

Hb = Hemoglobin is used for estimation; *Hb total; **Hb inferior and superior sectors; C/D = vertical cup-to-disc ratio; RNFLT = retinal nerve fiber layer thickness; GDF = glaucoma discriminant function; VF = visual field; MD = mean deviation; PSD = pattern standard deviation.
was observed than with the corresponding rim area (p = 0.02) or thickness of the fiber layer in this area (p < 0.0001; Figure 3).

There was good correlation between the values of vertical C/D ratio (r² = 0.67, p < 0.001) and rim area (r² = 0.62, p < 0.001) estimated from topographic relative Hb values, with those measured directly by Cirrus OCT (Figure 4).

The receiver operating characteristic (ROC) areas showed maximum values for OCT rim area and a combination of Laguna ONhE GFD with the perimetric indices, using the formula (GDF × 2) + (PSD × −5) + MD. However, the confidence intervals only presented overlapping with those of various morphological and functional parameters, such as OCT vertical C/D ratio, DM or GDF, so, with this sample, we did not find statistically significant superiority with respect to them, only when compared to others such as OCT thickness or PSD. A similar result was found when analyzing the 49 glaucoma suspects (Table 4).

DISCUSSION

Pallor of optic nerve head can be assessed by measuring tissue absorption of one or two wavelengths, but these estimates may not be accurate if an internal standard is not used to control the characteristics of the observation light, lens absorption and the spectral response of the detectors used. This is the main contribution of our method.

A new procedure should not be accepted based on a single experience. In a previous publication, we separated the results of cup and rim hemoglobin content using stereoscopic images. The results of that work indicate that the remaining rim had low perfusion in many cases, that perfusion was better related with perimetric results than with sectorial areas of the rim and finally, that the association of morphological and functional data may have some diagnostic advantages. In that article, we said that the results should be confirmed in an independent set of data. This was the intention of this study, which additionally addressed the segmentation of the cup and rim by a different process. Despite some differences, the results of the two studies can be considered concordant.

Segmentation provided by OCT Cirrus combined with photographs obtained with the fundus camera allowed the Laguna ONhE program to quantify the amount of hemoglobin present in the cup and in sectors of the rim separately.

Numerous studies have been performed to compare the relationship between visual field sensitivity and morphological indices, using confocal tomography (Heidelberg Retina Tomograph – HRT), scanning laser polarimetry (GDx) or optical coherence tomography (OCT). However, our results suggest that visual function in glaucoma depends more on the amount of hemoglobin that is supplied to the remnant nerve fibers than their thickness or area, endorsing what other studies have suggested. What needs to be clarified is whether reduced perfusion is what conditions subsequent tissue atrophy or is a consequence of this. The observation that in many cases the remaining rim of glaucoma cases has insufficient perfusion could support the first of these two hypotheses. Correlation of structure and function might be improved even further by selective perimetry.

Another question derived from the above is whether the amount of hemoglobin can provide prognostic information. For this, we would have to measure changes in IOP induced by medication or surgery, and to determine whether their values significantly reflect the process of visual field deterioration. At the present stage of our investigation, we have not performed any studies in the same population using Laguna ONhE and other methods which estimate perfusion.

However, it seems interesting to compare some of our data with those published recently by authors using the new technique of OCT angiography, taking into account that this is a preliminary study and involved only a limited number of cases.

First, the value of r² obtained on comparing perimetric MD with “Disc Flow Index” of OCT angiography was 0.138 (p = 0.146). In our case, the linear correlation between MD and the amount of Hb in the superior and inferior sections of the rim was r² = 0.401 (p < 0.0001). A logarithmic fit does not improve the result.

Second, the value of r² obtained on comparing the PSD with the “Disc Flow Index” of OCT angiography...
was 0.752 \( (p = 0.001) \). This result is not easily explained, because we know that PSD does not have a linear relationship with glaucoma progression; it increases in the initial stages and decreases in advanced stages. In our case, the correlation between PSD and the amount of Hb in the superior and inferior sections of the rim was \( r^2 = 0.153 \) \( (p < 0.0001) \).

Disc Flow Index showed low logarithmic correlation with C/D area \( (r^2 = 0.048, p = 0.273) \), while in our case, total Hb showed high linear correlation with OCT vertical C/D ratio \( (r^2 = 0.441, p < 0.0001) \).

Disc Flow Index showed good logarithmic correlation with rim area \( (r^2 = 0.397, p = 0.026) \), and in our case, total Hb also showed good correlation with rim area, but linearly \( (r^2 = 0.398, p < 0.0001) \).

Finally, Disc Flow Index had low correlation with logarithmic NFL thickness \( (r^2 = 0.004, p = 0.853) \), while in our case, total Hb showed significant linear correlation with this index \( (r^2 = 0.207, p < 0.0001) \).

We must insist that these data can only be considered a very preliminary estimate of the relative potential of the two procedures, which needs to be clarified by comparative studies of the same population sample.

Our results allow us to speculate that we may, in the future, in glaucoma do without the information provided by OCT to discriminate between intact rim and the remaining disc, since the distribution of relative Hb in the ONH allows a fairly good estimate of some morphological parameters such as rim area or

**FIGURE 3** Relationship between Hb amount, nerve fiber layer thickness and rim area in the superior and inferior sectors of the rim with respect to mean deviation (MD) of the corresponding sectors of the visual field.
vertical C/D ratio. This information may allow us to define the boundaries of the cup and the functional intact rim, aided by image segmentation methods. If so, we could obtain similar results to those presented here exclusively using conventional color images, not stereoscopic, and without the need for additional morphological measurement devices, which would greatly simplify the process and facilitate its use.

Other important issues for the future may be the specific assessment of normal pressure glaucoma or longitudinal studies to determine the prognostic value of the method and comparisons with other methods of perfusion analysis.

**DECLARATION OF INTEREST**

Marta Gonzalez-Hernandez and Manuel Gonzalez de la Rosa are partners of INSOFT SL which has patented the method. The remaining authors have no competing interests. This study was supported in part by

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**TABLE 4 Results of receiver operating characteristic (ROC) analysis: ROC area confidence intervals, sensitivity for a specificity of 95% and p value.**

<table>
<thead>
<tr>
<th></th>
<th>ROC AREA CI 0.95</th>
<th>ROC AREA CI 0.05</th>
<th>Sensitivity (Specificity 95%)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>OCT rim area</td>
<td>0.935</td>
<td>0.867</td>
<td>71.9</td>
<td>2.7E-33</td>
</tr>
<tr>
<td>(GDFx2) + (PSDx-5) + MD</td>
<td>0.934</td>
<td>0.865</td>
<td>75.2</td>
<td>2.6E-31</td>
</tr>
<tr>
<td>OCT C/D</td>
<td>0.926</td>
<td>0.854</td>
<td>70.2</td>
<td>5.6E-28</td>
</tr>
<tr>
<td>GDF</td>
<td>0.925</td>
<td>0.853</td>
<td>70.2</td>
<td>1.6E-29</td>
</tr>
<tr>
<td>Hb rim area</td>
<td>0.915</td>
<td>0.839</td>
<td>66.9</td>
<td>7.2E-28</td>
</tr>
<tr>
<td>Hb C/D</td>
<td>0.906</td>
<td>0.827</td>
<td>57.9</td>
<td>2.4E-26</td>
</tr>
<tr>
<td>MD</td>
<td>0.892</td>
<td>0.808</td>
<td>52.9</td>
<td>1.5E-17</td>
</tr>
<tr>
<td>OCT RNFLT</td>
<td>0.874</td>
<td>0.784</td>
<td>54.5</td>
<td>1.3E-20</td>
</tr>
<tr>
<td>PSD</td>
<td>0.873</td>
<td>0.784</td>
<td>47.9</td>
<td>1.5E-14</td>
</tr>
<tr>
<td>OCT rim area MD &gt; -3 dB</td>
<td>0.896</td>
<td>0.770</td>
<td>54.0</td>
<td>1.3E-31</td>
</tr>
<tr>
<td>(GDFx2) + (PSDx-5) + MD</td>
<td>0.892</td>
<td>0.763</td>
<td>59.2</td>
<td>2.6E-31</td>
</tr>
<tr>
<td>GDF</td>
<td>0.891</td>
<td>0.764</td>
<td>58.0</td>
<td>1.6E-29</td>
</tr>
<tr>
<td>OCT C/D</td>
<td>0.886</td>
<td>0.755</td>
<td>51.0</td>
<td>5.6E-28</td>
</tr>
<tr>
<td>Hb rim area MD &gt; -3 dB</td>
<td>0.875</td>
<td>0.741</td>
<td>37.5</td>
<td>2.4E-26</td>
</tr>
<tr>
<td>OCT RNFLT</td>
<td>0.768</td>
<td>0.611</td>
<td>24.5</td>
<td>1.4E-20</td>
</tr>
</tbody>
</table>

MD = mean deviation; C/D = vertical cup-to-disc ratio; GDF = glaucoma discriminant function; RNFLT = retinal nerve fiber layer thickness; PSD = pattern standard deviation; Hb = hemoglobin. CI = confidence interval.
*p* = Student t-test.

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**FIGURE 4 Relationship between vertical C/D ratio (left) and rim area (right) measured by OCT and estimated by hemoglobin distribution in the optic nerve head.**
grant PI12/02307 of the Instituto de Salud Carlos III with FEDER funds.

REFERENCES


