

# Retinal and Macular Ganglion Cell Count Estimated With Optical Coherence Tomography RTVUE-100 as a Candidate Biomarker for Glaucoma

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**PURPOSE.** To evaluate the ability of total and macular estimated retinal ganglion cell (RGC) counts to discriminate between healthy and glaucomatous eyes. To determine threshold markers of the estimated RGCs taking into account age dependence.

**METHODS.** This was a cross-sectional, observational study. The study group consisted of 176 eyes subdivided in three groups: 32 healthy, 91 preperimetric (PPG), and 53 primary open-angle glaucoma (POAG) eyes. The estimate of total and macular number of RGCs was obtained using a model described later. To account for the inverse correlation of RGC count with age, we considered two age subgroups ( $\leq 55$  and  $> 55$  years) for both total and macular estimated RGC counts. We computed frequency distributions and receiver operating characteristic (ROC) curves to measure the discriminating ability and derive the cut-offs between two different conditions with their relative diagnostic parameters.

**RESULTS.** The total and macular estimated RGC counts showed highly significant differences among the three groups ( $P < 0.0001$ ). The estimated RGC counts performed fairly well in distinguishing healthy from glaucomatous (PPG+POAG) eyes (area under the curve [AUC] = 0.79–0.92) with no statistically significant difference between total and macular RGCs. The approach allowed a good discrimination also between PPG and POAG eyes (AUC = 0.86–0.92). Cutoffs for the older age bracket were found to be lower in all cases.

**CONCLUSIONS.** Retinal ganglion cell counts estimated with empirical formulas with RTVue-100 could be used as a valid surrogate for neural losses in glaucoma.

**Keywords:** ganglion cells, glaucoma, optical coherence tomography

Glaucoma is an optic neuropathy, deriving from progressive retinal ganglion cells (RGC) loss, that causes thinning of retinal nerve fiber layer (RNFL) and progressive appearance of visual field defects.<sup>1</sup> Perimetry is currently used for diagnosis and staging of disease, but many studies showed that structural changes precede the loss of visual function and that a considerable number of RGCs is lost before the disease becomes clinically relevant.<sup>2–8</sup> For this reason, optical coherence tomography (OCT), enabling visualization of the retinal layers similar to the histologic appearance, is more and more frequently used to identify structural changes in the early stages of the disease. Recently, measurements of structural and functional tests were combined into empirical formulas for the estimation of RGCs.<sup>9–12</sup> A direct quantification of RGCs is not applicable in vivo, but it is possible to estimate the number of RGCs from empirical formulas combining standard automated perimetry (SAP) sensitivities and measurements of RNFL thickness by OCT.<sup>9,10</sup> These formulas have shown a valid correlation with histologic counts in monkeys' retinas in experimental glaucoma models.<sup>9</sup> These estimates seem to perform better than functional and structural parameters considered separately, both for staging and monitoring the disease.<sup>10–14</sup>

The previously demonstrated involvement of the macula<sup>15</sup> in early glaucoma is compatible with the fact that the macula is the retinal region with the highest density of RGCs.<sup>16,17</sup> As a matter of fact, 50% or more of total RGCs are contained within

16° of the foveal center, a region that represents 7.3% of the total retina.<sup>18</sup> The estimated count of the macular RGC number could be a useful parameter to identify the presence of glaucomatous damage.

The clinical applications of estimated RGC counts for glaucoma diagnosis have been published before.<sup>10–14</sup> We investigated a possible role of the estimated RGC count as a biomarker that is an objectively measurable feature with the ability to predict the risk of a disease,<sup>19</sup> or to indicate a pathologic biological process<sup>20</sup> when its value is beyond a certain threshold. As regards to glaucomatous optic neuropathy, such a biomarker could be used to identify patients with a higher risk of having glaucoma and to distinguish between the various stages of the disease.

The primary goal of this study was to evaluate the ability of total and macular estimated RGC counts to discriminate between healthy and glaucomatous eyes and between eyes in various stages of glaucomatous disease. The secondary goal was to determine threshold markers of the estimated RGC count taking into account the impact of aging.

## METHODS

This cross-sectional observational study was conducted at the Glaucoma Center of the Eye Clinic, Department of Surgical Sciences, University of Torino, Italy. The methods were applied in accordance to the tenets of the Declaration of Helsinki;



informed consent was obtained from all subjects and the Ethics Committee (University & General Hospital San Giovanni Battista of Torino) gave approval.

To be included in the study, participants had to meet the following criteria: age between 18 and 80 years, best-corrected visual acuity (BCVA) greater than or equal to 20/30, spherical equivalent greater than  $-5.0$  diopters (D) and less than  $+3.0$  D, and open angle on gonioscopy. Subjects with ocular surgery, retinal or macular pathology, or systemic or neurologic conditions that could produce visual field defects, were excluded.

All subjects underwent a comprehensive ophthalmic examination consisting of: BCVA, ultrasound pachymetry, slit-lamp biomicroscopy of anterior and posterior segment, Goldmann applanation tonometry (GAT), diurnal tonometric curve, gonioscopy, peripapillary, and macular imaging using Fourier Domain-OCT (FD-OCT RTVue-100 software version A4, 5, 0, 59; Optovue, Fremont, CA, USA). SAP was performed with Swedish Interactive Threshold Algorithm (SITA) Standard strategy, program 24-2 of the Humphrey Field Analyzer (HFA; Carl Zeiss Meditec, Jena, Germany). Fixation losses less than or equal to 20%, false positives and false negatives less than or equal to 33% were established as the reliability criteria.

The study included three groups of subjects: PPG, glaucomatous, and a control group recruited from the healthy population. The latter were required to have no family history of glaucoma, highest daily IOP less than 21 mm Hg, a normal visual field (VF) test and a normal optic nerve head (ONH) appearance. Patients affected by PPG had the highest daily IOP greater than 21 mm Hg, normal VF test, and ONH changes (cup-disc ratio alteration, disc hemorrhages, rim notching, diffused or localized RNFL defects). Primary open-angle glaucoma (POAG) was diagnosed based on the presence of highest daily IOP greater than 21 mm Hg, abnormal VF according to Hodapp-Parrish-Anderson criteria for diagnosing glaucomatous damage,<sup>21</sup> and glaucomatous optic disc changes. Optic nerve head appearance was evaluated by slit-lamp biomicroscopy of the posterior segment using a 78-D lens.

### FD-OCT RTVue-100

The Glaucoma Protocol of FD-OCT RTVue-100 was used to acquire RNFL thickness measurements. All scans were repeated three times by the same operator. The RNFL thicknesses used for the analysis derived from the scan with the highest signal strength index (SSI).

Optic nerve head scans consist of 13 circle lines with a diameter of 1.3 to 4.9 mm centered on the optic disc. These lines were used to create a peripapillary nerve fiber layer thickness (RNFL) map while 12 radial lines with 3.7-mm length were used to determine morphologic parameters of disc margin. This scan was used to calculate average, average-superior, and average-inferior RNFL thicknesses.

The three-dimensional (3D) disc protocol was used to identify the optic disc margin to obtain RNFL measurement. It consists of a  $5 \times 5$  mm square region with 101 horizontal lines and a total of 51,813 A-scans.

Scans with motion artifacts and with signal strength index less than 45 were excluded.

### Estimate of Retinal and Macular Ganglion Cell Count

The estimated of RGC count was obtained by applying the model developed and described in detail by Medeiros et al.<sup>10-12,22</sup> based on the empirical formulas processed by Harwerth et al.<sup>9</sup> In this model, data from structural and functional tests are elaborated to derive a final estimate of the RGC count. The number of RGC somas in a retinal area

corresponding to a specific SAP test field location at eccentricity ( $ec$ ) with sensitivity ( $s$ ) in dB is estimated through the following formula:

$$m = [0.054 \times (ec \times 1.32)] + 0.9 \quad (1)$$

$$b = [-1.5 \times (ec \times 1.32)] - 14.8 \quad (2)$$

$$gc = \left\{ \frac{[(s-1) - b]}{m} \right\} + 4.7 \quad (3)$$

$$SAPrgc = \sum 10^{(gc \times 0.1)} \quad (4)$$

In the formula,  $m$  and  $b$  represent the slope and the intercept of the linear function describing the relationship between the ganglion cell quantity ( $gc$ ) in decibels and the VF  $s$  in decibels at a given  $ec$ . The cell density obtained from each perimetry measurement is supposed to be almost uniform over a retinal region corresponding to an area of  $6^\circ \times 6^\circ$  of visual space that separates test locations in SAP. The SAP-derived estimated RGC count (SAPrgc) derives from the sum of the estimates in all SAP locations.

The number of RGC axons is estimated using the RNFL thickness measurement by OCT. The model considers the effect of aging on axonal density. The estimated number of RGC axons (OCTrgc) is obtained with the formula:

$$d = (-0.007 \times \text{age}) + 1.4 \quad (5)$$

$$c = (-0.26 \times \text{MD}) + 0.12 \quad (6)$$

$$a = \text{average RNFL thickness} \times 10870 \times d \quad (7)$$

$$\text{OCTrgc} = 10^{[(\log(a) \times 10 - c) \times 0.1]} \quad (8)$$

In the above formula,  $d$  is the axonal density (axons/ $\mu\text{m}^2$ ),  $c$  is a correction factor for the severity of disease taking into account remodeling of the RNFL axonal and nonaxonal composition, and MD is the SAP mean deviation. These formulas provide two estimates of the number of RGCs, one from a functional source and one from a structural source. Finally, the total estimated RGC count is obtained as:

$$\text{Estimated total RGC count} = \left( 1 + \frac{\text{MD}}{30} \right) \times \text{OCTrgc} + \left( \frac{-\text{MD}}{30} \right) \times \text{SAPrgc} \quad (9)$$

Estimated macular RGC count was calculated using the following formulas<sup>22</sup>:

$$m = [0.054 \times (ec \times 1.32)] + 0.9 \quad (10)$$

$$b = [-1.5 \times (ec \times 1.32)] - 14.8 \quad (11)$$

$$gc = \left\{ \frac{[(s-1) - b]}{m} \right\} + 4.7 \quad (12)$$

$$\text{Macular SAPrgc} = \sum 10^{(gc \times 0.1)} \quad (13)$$

The formula for macular SAPrgc is the same used for total RGCs estimate, but only 16 central points of the 24-2 SAP test were used; they correspond to the central  $10^\circ$ .

TABLE 1. Comparison of the Demographic and Clinical Characteristics of Healthy Eyes, PPG, and POAG Eyes

Parameters	Healthy	PPG	POAG	P Value
Eyes (n)	32	91	53	
Age (y)	54.34 (9.28)	55.6 (11.12)	57.40 (9.67)	0.31*
Sex (male/female)	10/22	30/61	21/32	0.67†
BCVA (decimal fraction)	0.93 (0.08)	0.94 (0.09)	0.91 (0.1)	0.14*
SE (D)	-0.6 (1.62)	-0.87 (2.11)	-0.68 (1.96)	0.74*
IOP (mm Hg)	15.38 (2.01)	17.22 (2.70)‡	16.26 (2.45) §	0.001*
MD (dB)	-0.26 (0.72)	-0.88 (1.28)	-5.17 (3.91)‡§	<0.0001*
PSD (dB)	1.67 (0.41)	1.70 (0.35)	4.64 (2.61)‡§	<0.0001*
RNFL average (µm)	109.26 (9.23)§	95.28 (10.65)‡	83.22 (11.03)‡§	<0.0001*
Estimated total RGC count	1,151,924 (113,441)§	969,506 (162,907)‡	654,706 (176,499)‡§	<0.0001*
Estimated macular RGC count	593,315 (65,834)§	507,036 (75,685)‡	352,047 (102,247)‡§	<0.0001*

The parameters are expressed as mean and SD. SE, Spherical Equivalent; IOP (highest value of the diurnal tonometric curve); MD of SAP; PSD of SAP.

\* ANOVA.

†  $\chi^2$  test.

‡ Statistically significant difference from healthy eyes group (LSD post hoc test).

§ Statistically significant difference from PPG eyes group (LSD post hoc test).

Temporal peripapillary RNFL thickness measured by OCT is entered in the following formula in order to estimate the number of macular RGC axons:

$$d = (-0.007 \times \text{age}) + 1.4 \quad (14)$$

$$c = (-0.26 \times \text{macular TD}) + 0.12 \quad (15)$$

$$a = \text{average temporal RNFL thickness} \times 0.5 \times 10870 \times d \quad (16)$$

$$\text{Macular OCTrgc} = 10^{[(\log(a) \times 10 - c) \times 0.1]} \quad (17)$$

The macular total deviation (macular TD), used instead of MD, corresponds to the average TD of the central 16 points of 24-2 SAP in decibels.<sup>22</sup> Thus, the macular estimated RGC count is obtained with the same formula used for total RGC count:

$$\begin{aligned} \text{Estimated macular RGC count} = & \left(1 + \frac{\text{MD}}{30}\right) \times \text{Macular OCTrgc} \\ & + \left(\frac{-\text{MD}}{30}\right) \times \text{Macular SAPrgc} \end{aligned} \quad (18)$$

## Statistical Analysis

Continuous variables were checked to meet the normality conditions of Shapiro-Wilk test. Analysis of variance and Fisher's least significant difference (LSD) post hoc test with Bonferroni adjustment were used for intergroup comparisons for normally distributed variables. Dichotomic variables were arranged in cross-correlation tables and studied with a  $\chi^2$  test or Fisher exact test.

The study of the frequency distributions of the pathologic and healthy global and macular estimated RGC count allowed to compute the level-specific likelihood ratios (LR) defined as the ratio of the frequency of the pathologic condition to the frequency of the healthy one (or more severe to less severe): the value of RGC count for which level-specific LR = 1 marks the border between dominance of one condition over the other.

The frequency distributions were the basis for computing the receiver operating characteristic (ROC) curve, that is the

plot of true positive rates (sensitivity; SNS) versus false positive rates (1-specificity; SPC): the area under the curve (AUC) is the standard parameter, which quantifies the ability of discriminating between two conditions, AUC = 0.5 being null (chance) and then increasing from 0.6 (poor) to 1 (excellent).

The values of SNS and SPC characterizing each level of RGC count allow deriving various parameters. They determine the associated LR of a positive test LR+ = SNS/(1-SPC) (i.e., the likelihood that a positive test result would be expected in a patient with disease compared with the likelihood that the same result would be expected in a patient without disease). Positive LRs, being the ratio of posttest odds to pretest odds, allows to estimate the posttest probability after a diagnostic test: LR+ greater than 1 produces a posttest probability of a positive result for a target disorder, which is higher than the pretest probability (i.e., rules in favor of disease). Sensitivity and SPC also identify the most appropriate cutoff between the two target conditions, through maximization of: (1) harmonic mean (HM) of SNS and SPC, (2) Jouden's index J = SNS + SPC - 1, and (3) Cohen's kappa, and minimization of the distance of the curve from the (0, 1) upper left vertex  $D = ((1 - \text{SNS})^2 + (1 - \text{SPC})^2)^{0.5}$ . This cutoff value is of course coincident with the one identified by the frequency distributions in correspondence to level specific LR = 1, but it has the added value of the quantification of the discriminating ability.

Differences between the AUCs were tested for statistical significance with the method of Hanley and McNeil.<sup>23</sup>

Correlation between continuous variables was estimated using the Pearson linear coefficient and regression analysis.

Statistical significance was set at *P* less than or equal to 0.05. The SPSS program package version 19.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

## RESULTS

The study group consisted of 176 eyes: 32 healthy, 91 PPG, and 53 POAG eyes. Demographic characteristics of the study population are illustrated in Table 1.

The ANOVA and  $\chi^2$  test showed that the groups are comparable for age, sex, visual acuity, and refraction. Intraocular pressure has a lower mean value for POAG eyes than for PPG eyes; this is justified by the fact that not every PPG eye received antiglaucomatous therapy. As expected, glaucomatous eyes have significantly worse VF MD than healthy and PPG eyes (*P* < 0.0001), while there is no

statistically significant difference between healthy and PPG eyes in regard to the MD value ( $P = 0.2$ ).

The total and macular estimated RGCs show highly significant differences among the three groups ( $P < 0.0001$ ). The estimated macular RGC counts represent approximately 52% of total RGC in healthy eyes, 53% in PPG eyes, and 54% in POAG eyes. Compared with the estimated total number of RGCs in healthy eyes, POAG eyes have 43% fewer estimated RGCs and PPG eyes have 16% fewer total RGCs. Similar proportions hold also for the mean macular estimated counts (41% and 15%).

The estimated RGC number has a moderate but significant linear inverse correlation with age in all three groups. For the healthy group (the simplest case, where the only independent variable is age and no other factors linked to the loss of RGCs) we derived a Pearson's coefficient  $r = -0.46$  ( $P = 0.007$ ) and regression coefficient  $b = -5625$  RGCs/year, corresponding to a physiological average loss of approximately 50,000 to 60,000 total estimated RGC counts every 10 years.

On the average, it is thus to be expected that also the cutoff between healthy and pathologic conditions will be affected by the aging process. To check this point within the limits posed by the individual peculiarities and the small sample size of the healthy group, we considered two subgroups, using as divider the median age of patients: 55 years or younger (healthy  $n = 24$ , PPG  $n = 39$ , POAG  $n = 10$ ) and older than 55 years (healthy  $n = 8$ , PPG  $n = 52$ , POAG  $n = 43$ ).

The Figure shows on the left the results for the total estimated RGC count for patients older than 55 years when testing the discriminating ability between healthy and glaucomatous eyes (PPG+POAG). The frequency distributions (Fig. A) share a modest region of overlapping, reflected in the good value of AUC (0.92) for the ROC curve (Fig. B). The four methods for threshold determination (Fig. C) unanimously confirm that the value RGC = 900,000 is the most appropriate threshold between glaucoma and not glaucoma (dotted line). At cutoff, sensitivity is 100%, specificity 73% and the LR of positive test LR+ is 3.7.

The results of the analogous procedures on the total estimated RGC count for patients younger than 55 years are shown in the first line of Table 2; the first two lines of Table 3 report all corresponding results for the macular estimated RGC count.

In order to analyze also the capability of estimated RGC count in differentiating between PPG and POAG eyes, we repeated the analysis comparing the two PPG and POAG groups. The results for patients older than 55 years are shown on the right side of the Figure: frequency distributions (Fig. D), ROC curve (Fig. E), and threshold determination (Fig. F). We can see that AUC = 0.90 and the cutoff value is RGC = 800,000, with 77% SNS, 88% SPC, and LR+ = 6.6. The results of the analogous procedures on the total estimated RGC count for patients younger than 55 years, are shown in the third line of Table 2; Table 3 reports all corresponding results for the macular estimated RGC count. For completeness, the tables report also the outcomes of the analysis on healthy versus PPG and healthy versus POAG for both total and macular estimated RGC counts.

Generally, the AUC values do not show significant differences between macular and total estimated RGC; the same is true also for the comparison between the two age brackets.

## DISCUSSION

In the present study, we explored the possibility of using RGC counts estimated from structural and functional mea-

surements in distinguishing glaucomatous from healthy eyes in an independent population. In fact, several studies demonstrated that the use of a combined structure and function measure to estimate RGC losses performed better than isolated structural and functional parameters. The study carried out by Medeiros et al.<sup>14</sup> attested that estimated RGC counts performed better than average RNFL thickness in discriminating glaucomatous from healthy eyes (AUC 0.95 vs. 0.88, respectively). Zhang et al.<sup>22</sup> outlined the superiority of macular and total estimated RGC count in comparison to macular ganglion cell-inner plexiform layer (mGCIPL) thickness and RNFL thickness, respectively (AUC 0.87 vs. 0.78 for macular parameters and 0.93 vs. 0.86 for total RNFL parameters). Our study was based on these assumptions and was meant as a validation of an established method for estimating the RGC count using a different FD-OCT (RTVue-100).

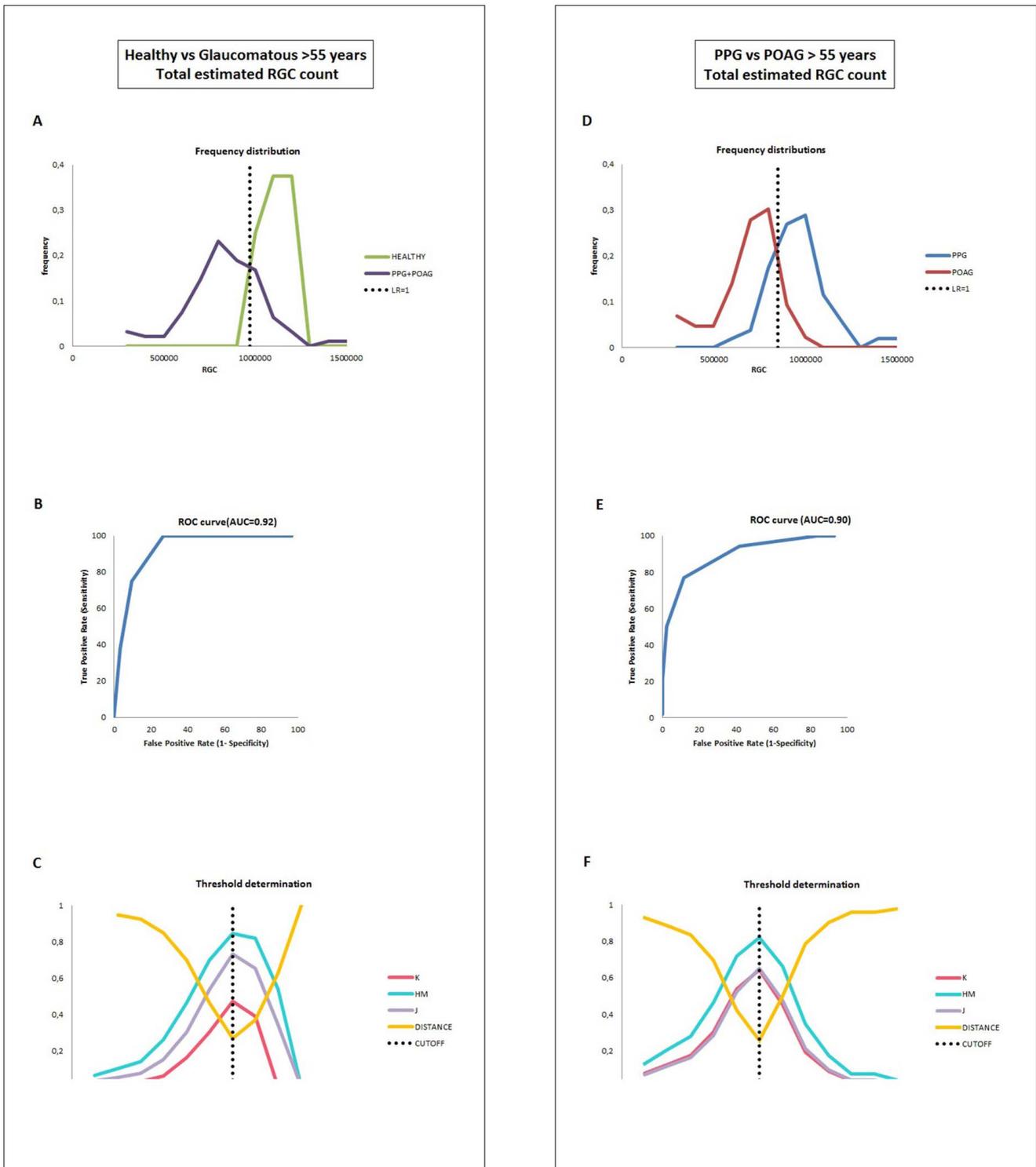
Our results indicated that the estimated RGC count of the healthy eyes group ( $1,151,924 \pm 113,441$ ) was consistent with the number of RGCs observed in studies on histologic sections of the optic nerve.<sup>24-26</sup> The total number of RGCs in the optic nerves of healthy donors was found to vary from 969,279 in the study conducted by Mikelberg et al.<sup>24</sup> to 1,244,005 in the study of Balazsi et al.<sup>25</sup> Also, our total number of estimated RGC in glaucomatous eyes was similar to that found in other studies with comparable MD SAP values, thus validating the approach of estimating the RGC count using different types of OCT instrument (654,706 RGCs, 687,219 RGCs, and 570,433 respectively, in our study, in Marvasti et al.<sup>13</sup> and in Zhang et al.<sup>22</sup>).

Because both total and macular estimated RGCs have a significant linear inverse correlation with age, a thorough analysis should take this factor in consideration when searching for cutoffs between pathologic and healthy conditions. This issue would call for an extremely articulate approach: as an example, for the healthy group ( $r = -0.46$ ) the regression coefficient suggests an average loss of approximately 5000 to 6000 total RGC counts per year, but there are two patients separated by more than 30 years of age (45 vs. 76 years) who share the same RGC count. The awareness of the biological variability and of the peculiarity of each individual is well known to challenge any generalization; at the same time the use of paradigms and brackets of values are useful stepping-stones for most diagnostic paths. All considered, including the reduced sample size of our healthy group we chose as feasible option a rough subdivision in two age brackets ( $\leq 55$  and  $> 55$  years).

The estimate of RGC count with the RTVue-100 performed fairly well in distinguishing healthy from glaucomatous (PPG+POAG) eyes in both age brackets (AUC = 0.80-0.92 for the total estimated RGCs and AUC = 0.79-0.81 for the macular estimated RGCs two age brackets) with no statistically significant difference between total and macular estimated RGCs.<sup>22</sup> Our results are similar to the AUC values found by Zhang et al.<sup>22</sup> relative to all ages (AUC 0.93 and 0.87 for the total and macular estimated RGC counts).

The approach showed a good discrimination also between PPG and POAG eyes (AUC = 0.86-0.92) for all ages and for both total and macular estimated RGC counts.

As expected, the discrimination between healthy and PPG eyes was more arduous: the AUC for total estimated RGC count ranged from 0.75 for ages below 55 years to 0.83 for ages above 55; for macular estimated RGC count, from 0.74 to 0.69. The relatively lower ROC curve areas for these groups may reflect the inaccuracy of PPG diagnosis. Some of the PPG eyes could be actually normal; diagnosis of PPG is based on biomicroscopy of the ONH in the absence of VF loss, thus it would require longitudinal follow-up for diagnostic confirmation.



**FIGURE.** On the *left*, the results obtained for the total estimated RGC count for patients older than 55 years when testing the discriminating ability between healthy and glaucomatous eyes (PPG+POAG). On the *right*, the results obtained for the total estimated RGC count for patients older than 55 years when testing the discriminating ability between PPG and POAG eyes. (A, D) Estimated RGC frequencies distributions. (B, E) ROC curves. (C, F) Threshold determination.

The inverse relation between age and RGCs number did not significantly affect the discriminating ability of the test, the comparison between the AUC values for the two brackets leading to *P* values well above 0.05. The impact was instead important on the determination of the cutoff values: the cut-offs for people older than 55 years were found to be lower for

all cases. This means that a given total estimated RGC count (for instance 1,000,000) lays below the 1,100,000 threshold for glaucomatous (PPG+POAG) eyes for a person younger than 55 years but above the 900,000 threshold for an older one. The patient below 55 with RGC count =1,000,000 has a LR for positive test  $LR+ = 3.3$ , approximately 50% higher than the

**TABLE 2.** Results of the ROC Curve Procedure for the Total Estimated RGC Count: AUC With 95% Confidence Intervals (95%CI) and Cutoff Values With Relative SNS, SPC, and LR+

Groups	Estimated Total RGC Count				
	AUC (95%CI)	Cutoff (RGCs)	SNS (%)	SPC (%)	LR+
Healthy vs. PPG+POAG					
≤55 y	0.80 (0.69-0.90)	1,100,000	70.83	70.83	2.4
>55 y	0.92 (0.86-0.99)	900,000	100.00	73.12	3.7
PPG vs. POAG					
≤55 y	0.92 (0.82-1)	900,000	87.18	80.00	4.4
>55 y	0.90 (0.83-0.96)	800,000	76.92	88.37	6.6
Healthy vs. PPG					
≤55 y	0.75 (0.63-0.87)	1,100,000	70.83	64.10	2.0
>55 y	0.83 (0.71-0.94)	1,000,000	75.00	78.85	3.5
Healthy vs. POAG					
≤55 y	0.99 (0.95-1)	1,000,000	100.00	90.00	10.0
>55 y	0.99 (0.98-1)	900,000	100.00	97.67	42.9

value 2.4 at the RGC = 1,100,000 cutoff (i.e., a strong indication for disease); the patient above 55 years with the same RGC count has LR+ = 2.1, approximately 43% lower than the value 3.7 at the cutoff, that is, even if we cannot radically exclude disease (the healthy-disease distributions have a region of overlapping) we can downgrade its possibility.

The LR+, greater than 2 at cutoff and increasing up to two-digit numbers as RGC values go beyond it, indicate the appropriateness of this test and its contribution to the diagnostic process for clinical decision-making in glaucoma. In this sense, the estimated RGC count could work as a biomarker.

Actually, the potential clinical applicability of the estimation of RGC cells was already assessed by other studies, which proved the usefulness of estimated RGC count for the diagnosis, staging, and progression detection of the glaucomatous damage. Gracitelli et al.<sup>27</sup> used estimates of RGC counts obtained by the same method used in the current study to demonstrate that eyes with disc hemorrhages showed faster rates of RGC loss compared with eyes without disc hemorrhages. The estimation of RGC cells was able to approximately establish the intereye difference in RGCs number associated with relative afferent papillary defect (RAPD).<sup>28</sup> Meira-Freitas et al.<sup>29</sup> showed that baseline and longitudinal estimates of RGC

counts could predict future developments of VF loss or optic disc damage in glaucoma suspect eyes. Patients with glaucomatous progression during follow-up were detected with higher accuracy using the estimated RGC count rather than functional and structural evaluations separately considered.<sup>29</sup> Furthermore in distinguishing glaucomatous from healthy eyes, RGC count demonstrated to perform better than cup-to-disc ratio (CDR), which has high intra- and interexaminer variability and a relative insensitivity to evaluate glaucomatous progression.<sup>30</sup> The estimated RGC count has also demonstrated the large RGC loss associated with localized RNFL defects visible on stereophotographs, usually considered one of the earliest changes of POAG.<sup>31</sup>

The estimated RGC count may have a role also in the interpretation of glaucomatous damage in the early stages. There is evidence that a considerable amount of RGC loss corresponds to a minimum change in visual field index (VFI) in early stages of the disease. Marvasti et al.<sup>13</sup> found that a change of 0.3% in VFI in eyes with initial VFI of 100% would correspond to a loss of 100,000 RGCs. Therefore, VFI underestimates real neural loss, especially in initial phases of disease. The results obtained in this study underline how a change of 100,000 RGCs not only changes the probability of disease, but also that the entity of the change varies with the

**TABLE 3.** Results of the ROC Curve Procedure for Macular Estimated RGC Count: AUC With 95% CI, Cutoff Values With Relative SNS, SPC, and LR+

Groups	Estimated Macular RGC Count				
	AUC (95% CI)	Cutoff (RGCs)	SNS (%)	SPC (%)	LR+
Healthy vs. PPG+POAG					
≤55 y	0.79 (0.68-0.90)	600,000	54.17	89.90	5.4
>55 y	0.81 (0.69-0.93)	500,000	75.00	77.89	3.4
PPG vs. POAG					
≤55 y	0.88 (0.76-0.99)	500,000	76.92	90.00	7.7
>55 y	0.86 (0.79-0.93)	400,000	88.46	72.09	3.2
Healthy vs. PPG					
≤55 y	0.74 (0.62-0.87)	600,000	54.17	87.18	4.2
>55 y	0.69 (0.51-0.87)	500,000	75.00	61.54	2.0
Healthy vs. POAG					
≤55 y	0.96 (0.90-1)	500,000	95.83	90.00	9.6
>55 y	0.95 (0.89-1)	500,000	75.00	97.67	32.2

estimated RGC number involved. Let us consider as an example three patients over 55 years, for whom the divider between healthy and pathologic conditions is 900,000 RGCs. Let us further assume that the three patients have similar VFI on SAP (100%, 97%, and 94%), but the first one scores 950,000 cells, the second 850,000, and the third one 750,000. The 100,000 RGCs difference between the first and the second patient corresponds to an increase of LR+ from 2.7 to 5.8 (a 3-fold increase of the probability of disease), whereas the very same cells difference between the second and the third patient corresponds to an increase of LR+ from 5.8 to 58 (a 10-fold increase).

Considering the relationship between VF and RGC loss, our study demonstrated that glaucomatous damage detectable with SAP arises at an average percentage of 43% total estimated ganglion cell loss and 41% macular estimated RGC loss. These percentages are consistent with the conclusions of other studies; Harwerth et al.<sup>32</sup> stated that the percentage ganglion cell loss associated with early VF defects is 50%, showing that a 20% to 50% loss of ganglion cells is required to produce a 6 dB loss by static perimetry.<sup>33</sup> According to other studies, at least 25% to 35% RGC loss is associated with abnormalities in automated VF testing.<sup>34,35</sup> However, some patients have changes detected on visual field prior to detectable structural changes and for this reason a combination of structural and functional assessments are needed to provide optimal diagnosis of glaucoma.

Our study has some limitations. The number of RGCs is not based on histologic data but is empirically derived from formulas. However, such an estimation of RGC count was shown to agree with histology in experimental glaucomas.<sup>9</sup> We are aware that the validation study of the formula for estimating RGC number used time-domain OCT,<sup>9</sup> however subsequent studies applying FD-OCT assessed the accuracy and validity of the formulas.<sup>10-14</sup> A possible cause of inaccuracy in estimating the total number of RGCs with the formula described by Medeiros et al.<sup>10</sup> lies in the different areas analyzed through OCT and SAP. Average RNFL thickness (measured by OCT) takes into account the entire region around the optic disc, corresponding to the RGCs of the entire retina; the 24-2 VF, on the contrary, analyses approximately  $\pm 27^\circ$  of visual angle from the fovea.<sup>36</sup> This disagreement is consistent with the conclusions of Raza and Hood.<sup>37</sup> This limitation, however, may not preclude the clinical usefulness of the method, as demonstrated by the present study and by several others.<sup>10-14,27-31</sup> The estimate of macular RGCs underlines a further limitation: the macular estimated RGC count could be affected by macular diseases. An accurate macular examination is therefore necessary to exclude any macular affection. Another limitation of the study was the use of the median value of 55 years for dividing our population in two age brackets.

In conclusion, our study confirms that RGC count estimated with empirical formulas with RTVue-100 can be used as a valid surrogate for neural losses in glaucoma. The determination of the threshold markers of the estimated RGC number and the use of LRs that take into account the age, could improve early diagnosis and management of glaucoma. The results of the present study, added to the considerable amount of previous works substantiating the clinical applicability, suggest that the estimated RGC count could well be taken into consideration as candidate biomarker for diagnosis and early detection of glaucoma progression.

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