


# Diurnal Stability Of Peripapillary Vessel Density And Nerve Fiber Layer Thickness On Optical Coherence Tomography Angiography In Healthy, Ocular Hypertension And Glaucoma Eyes

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**Purpose:** By using optical coherence tomography angiography (OCTA), to evaluate diurnal changes of the following parameters: vessel density in the peripapillary area (PP-VD) and in the optic nerve head (ONH-VD); thickness of retinal nerve fibre layer (RNFL) and ganglion cell complex (GCC).

**Methods:** All prospectively enrolled participants were imaged on the same day at 8 am and 7 pm by means of the XR Avanti device with AngioVue and AngioAnalytics software (Optovue Inc., Fremont, CA, USA). Only eyes with correct automatic segmentation and good-quality images (scan quality > 7/10) were included.

**Results:** Forty-six eyes from 28 patients with glaucoma, 53 from 31 patients with ocular hypertension, and 62 from 38 controls were assessed. The mean measurements of all parameters investigated were significantly different in the morning and in the evening values in all three groups, with lower values in glaucomatous eyes ( $p < 0.014$ ). In control eyes, as well as in eyes of patients with ocular hypertension and glaucoma the daily changes were minimal and not statistically significant with means of +0.17%, -0.31% and -0.54% for ONH-VD ( $p = 0.83$ ) and means of +0.2%, +0.1% and +0.29% for PP-VD ( $p = 0.83$ ), respectively. Likewise, daily fluctuations in GCC and RNFL thickness were not significant in any of the three groups, with means of +0.16  $\mu\text{m}$ , +0.66  $\mu\text{m}$  and -0.15  $\mu\text{m}$  for GCC ( $p = 0.58$ ) and means of +0.24  $\mu\text{m}$ , +0.58  $\mu\text{m}$  and -0.24  $\mu\text{m}$  for ONH-RNFL ( $p = 0.90$ ), respectively. Systemic hypertension, age, axial length, daily changes in intraocular pressure or scan quality did not correlate with daily fluctuations of any of the parameters investigated ( $p > 0.1$ ).

**Conclusion:** Vessel density, GCC and RNFL were stable during daytime thus corroborating the clinical relevance of OCTA regardless the time of acquisition.

**Keywords:** glaucoma, optical coherence tomography angiography, vessel density, daily fluctuations, retinal fiber layer

## Introduction

Glaucoma is a complex and multifactorial disease that involves loss of neural tissue, activation of glial cells, tissue remodeling and changes in blood flow. There is mounting evidence that vascular factors are involved, and ocular blood flow reduction probably plays a role in the pathogenesis of the optic nerve neuropathy associated with glaucoma.<sup>1,2</sup>

In recent years the use of optical coherence tomography angiography (OCTA) has become common in clinical practice, and it has exhibited good sensitivity and

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reproducibility in studies of retinal and papillary microcirculation without the intravenous injection of dye. Commercially available OCTA devices use different types of algorithms and technology and can yield elaborate quantitative maps of vascular density (VD) and thickness of the retinal area under investigation.<sup>3</sup> It has been suggested that tissue microvascular perfusion may be related to VD as measured by OCTA. Some evidence of reduced perfusion in the superficial optic nerve head (ONH) and peripapillary retina of individuals with primary open-angle glaucoma has been reported.<sup>4-8</sup> However, quantitative diurnal fluctuations have not been thoroughly investigated. As with the blood perfusion of other vascular regions such as the limbs, brain and digestive system<sup>9</sup> it is likely that ocular vascularization is affected by a circadian rhythm or is variably influenced by several factors. Although these effects may be temporary, understanding the alterations in optic nerve perfusion and in thickness of retinal nerve fibre layers (RNFL) or ganglionar cell complex (GCC) warrants further investigation, and may help clarify the aspects of glaucoma onset or progression.

The current study aimed to evaluate quantitative diurnal fluctuations in peripapillary and ONH vessel density, and in RNFL and GCC thickness in three groups: a group of patients with glaucoma; a group of patients with ocular hypertension; a control group. Relationships of these fluctuations with intraocular pressure (IOP) changes, axial length, systemic hypertension, and age were also investigated.

## Methods

### Study Population

Twenty-eight patients with glaucoma, 31 with ocular hypertension without visual field deterioration, and 38 healthy controls were recruited prospectively from January 2018 to November 2018 by the Department of Ophthalmology of the IRCCS Auxologico Institute, Milan, Italy. The study was approved by the relevant institutional review board of the IRCCS Auxologico Institute and it was conducted in accordance with the Declaration of Helsinki. Informed written consent was acquired from each patient.

The following additional inclusion criteria were applied in the glaucoma group: IOP  $\geq 21$  mmHg on three consecutive visits prior to IOP-lowering topical treatment, Repeatable glaucomatous visual field damage defined as a glaucoma hemi field test (GHT) outside normal limits and a pattern standard deviation outside 95% normal limits; glaucomatous visual field abnormalities

defined by a cluster of +3 adjacent points in the pattern deviation plot with a probability of  $< 5\%$  including at least 1 point with a probability  $< 1\%$  in at least two repeatable and consecutive standard automated perimetry tests.

The following additional inclusion criteria were applied in the ocular hypertension group: IOP  $\geq 21$  mmHg on three consecutive visits prior to IOP-lowering topical treatment, normal-appearing optic disc and intact neuroretinal rim on clinical examination defined as a vertical cup-to-disc ratio  $< 0.5$  and a vertical cup disc ratio asymmetry  $\leq 0.2$  with a minor vertical cup disc ratio  $\leq 0.4$ , at least two normal visual fields defined as a pattern standard deviation within 95% confidence limits and a GHT result within normal limits, and average and quadrant RNFL thicknesses within respective confidence limits of 95% and 99%.

The control group consisted of individuals without ocular hypertension, presenting the following characteristics: IOP  $< 21$  mmHg without therapy, no history of elevated IOP, normal-appearing optic disc and intact neuroretinal rim on clinical examination defined as a vertical cup-to-disc ratio  $< 0.5$  and a vertical cup disc ratio asymmetry  $\leq 0.2$  with a minor vertical cup disc ratio  $\leq 0.4$ , normal visual fields defined as a pattern standard deviation within 95% confidence limits and a GHT result within normal limits, and average and quadrant RNFL thicknesses within respective confidence limits of 95% and 99%.

The presence of systemic hypertension, cardiovascular diseases, and other diseases was recorded but not considered as exclusion criteria in any group. On the contrary, patients with diabetes were excluded.

### Study Design And Imaging Acquisition

All study participants underwent a complete ophthalmologic examination including best-corrected visual acuity measurement, slit-lamp biomicroscopy, IOP measurement with Goldmann applanation tonometry at the same day at 8 a.m. and 7 p.m., ultrasound pachymetry, gonioscopy, and dilated fundus examination. The axial length was measured in every eye using an IOL master 500 system (Carl Zeiss Jena, Germany). Participants also underwent standard automated perimetry tests (24-2 Swedish Interactive Threshold Algorithm; Humphrey Field Analyser II; Carl Zeiss Meditec, Inc., Dublin, CA, USA) and OCTA. The OCTA system used was the XR Avanti device with AngioVue and AngioAnalytics (software version 2017.1.0.150, Optovue Inc., Fremont, CA, USA), which enables visualization of

the retinal and papillary circulation by means of a split-spectrum amplitude decorrelation angiography algorithm. The instrument has an A-scan rate of 70,000 per second, using a light source centred on 840 nm.

The following three scan acquisition protocols were used at 8 a.m. and 7 p.m.:

- The HD Angio Disc 4.5 x 4.5 micrometers acquisition consists of two orthogonal OCTA volumes centered on the ONH and containing 400x400 scans to perform motion correction and minimize motion artifacts arising from microsaccades and fixation changes. Vascularity data are automatically converted to a VD map, where VD is calculated as the percentage area occupied by blood vessels. The percentage is provided and calculated automatically for the whole enface image (W-VD), for the optic disc only (ONH-VD) and for the peripapillary region defined as a 750- $\mu$ m-wide elliptical annulus extending from the optic disc boundary (PP-VD) and divided in the hemi-superior (PPHalfSup-VD) and hemi-inferior (PPHalfInf-VD) sectors. Eight sub-sectors of the peripapillary area are also calculated; the nasal-superior (PPNS-VD), nasal-inferior (PPNI-VD), inferior-nasal (PPIN-VD), inferior-temporal (PPIT-VD), temporal-inferior (PPTI-VD), temporal-superior (PPTS-VD), superior-temporal (PPST-VD) and superior-nasal (PPSN-VD) sub-sectors. The map obtained refers to the superficial capillary plexus of the ONH, which is also known as radial peripapillary capillary segmentation. It extends from the ILM to the outer limit of the RNFL and is composed of the vasculature of the RNFL and GCC layer.
- The macula scanning protocol was used to measure the GCC (i.e. the combined thickness of the RNFL, ganglion cell layer, and IPL) thickness. The centre of the GCC scan is automatically shifted 0.75 mm temporally to improve sampling of the temporal periphery. The resulting map shows the averaged thickness in micrometres of the whole W-GCC and of the hemi-superior (HalfSup-GCC) and hemi-inferior sector (HalfInf-GCC).
- To measure the RNFL thickness the standard glaucoma protocol was used with ONH map acquisition. The map is centred on the ONH along a circle of 3.45 mm in diameter and provides measurements in micrometres of RNFL for the whole, averaged ONH (ONH-RNFL) and for each of the 8 corresponding sub-sectors; the nasal-superior (NU-RNFL), nasal-lower (NL-RNFL),

inferior-nasal (IN-RNFL), inferior-temporal (IT-RNFL), temporal-lower (TL-RNFL), temporal-upper (TU-RNFL), superior-temporal (ST-RNFL) and superior-nasal (SN-RNFL) sub-sectors. The hemi-superior (HalfSup-RNFL) and hemi-inferior (HalfInf-RNFL) RNFL thicknesses are also calculated automatically.

Only eyes with correct automatic segmentation and disc margin location as controlled by the authors were included in the study. Only good-quality images—defined by scans with scan quality > 7/10 (and without artefacts)—were included.

## Statistical Analysis

Continuous variables are reported as means and standard deviations (SDs) or median and interquartile range (IQR) if variables are not normally distribute; categorical variables are reported as frequencies and percentages. The chi squared test was used to evaluate associations between patient-level categorical variables (systemic hypertension and sex) and group (healthy, ocular hypertension and glaucoma); age effects were assessed via analysis of variance (ANOVA); the change in intraocular pressure were assessed using a repeated measure ANOVA and the scan quality using the Kruskal-Wallis test

Three multivariate repeated measures ANOVA with an unstructured covariance matrix were applied to evaluate the differences in VD, GCC and RNFL thickness parameters means between groups accounting for the correlation between measure on eyes within the same patients. The response variables were the measurement of the ocular parameters in the morning, in the evening and the daily change whereas the independent variables were the group, systemic hypertension, age, axial length, daily changes in intraocular pressure and the scan quality. Tukey's post-hoc test was applied to pairwise comparisons between groups only for ocular parameter where a statistical difference between groups was found. Given the large number of tests performed, false discovery rate (FDR) adjustment was applied to the ANOVA test to account for the inflation of type I error due to multiple tests.

The analyses were performed using SAS (SAS Institute, Cary, NC USA), all tests were two tailed, and a p-value  $\leq 0.05$  was considered statistically significant.

## Results

Forty-six eyes from 28 patients with glaucoma (mean age  $59.54 \pm 7$  years), 53 eyes from 31 patients with ocular

hypertension (mean age  $60 \pm 7$  years) and 62 eyes from 38 controls (mean age  $57 \pm 6$  years) were included in this study. Nineteen patients were excluded because of the poor quality of the images. There were no significant differences in age, gender, axial length, best-corrected visual acuity, refractive status or presence of systemic hypertension among the three groups (Table 1,  $p > 0.14$ ). There was a significant difference pertaining to IOP ( $p < 0.0001$ ), which did not change significantly throughout the day ( $p = 0.59$ ). Forty-three eyes (26 patients) were taken topical prostaglandins, 34 eyes (21 patients) topical betablockers, 17 eyes (10 patients) carbonic anhydrase inhibitors and 4 eyes (two patients) brimonidine.

The mean measurements of all the parameters investigated were statistically different in the three groups in the morning and in the evening ( $p < 0.014$ , Table 2).

In control eyes, eyes with ocular hypertension and eyes with glaucoma the percentages of daily changes were minimal and not statistically significant, with respective means of  $+0.12\%$ ,  $+0.34\%$  and  $-0.25\%$  for W-VD (Table 2, FDR-adjusted  $p = 0.83$ );  $+0.17\%$ ,  $-0.31\%$  and  $-0.54\%$  for ONH-VD (FDR-adjusted  $p = 0.83$ );  $+0.2\%$ ,  $+0.1\%$  and

$+0.29\%$  for PP-VD (FDR-adjusted  $p = 0.83$ ). Daily fluctuations in GCC and RNFL thickness were not significant too in any of the three groups, with respective means of  $+0.16 \mu\text{m}$ ,  $+0.66 \mu\text{m}$  and  $-0.15 \mu\text{m}$  for GCC (Table 2, FDR-adjusted  $p = 0.58$ ) and  $+0.24 \mu\text{m}$ ,  $+0.58 \mu\text{m}$  and  $-0.24 \mu\text{m}$  for ONH-RNFL (FDR-adjusted  $p = 0.91$ ).

None of the parameter investigated changed significantly in the group by group analysis or when the total sample was considered (Table 3,  $p > 0.15$ ).

In the group to group comparison, the post-hoc Tukey's tests showed a statistically significant difference for every parameter investigated between eyes with glaucoma and control eyes or eyes with ocular hypertension (Table 4,  $p < 0.008$ ), except for ONH-VD and NL-RNFL in the evening ( $p > 0.08$ ). Comparisons between eyes with ocular hypertension and control eyes were not statistically significant ( $p > 0.17$ ).

Table 5 lists the regression coefficients and corresponding standard errors with FDR-adjusted  $p$  values derived from the multivariate model. Systemic hypertension, age, axial length, daily changes in IOP and scan quality were not significantly associated with daily fluctuations in VD, RNFL or GCC ( $p > 0.1$ ).

**Table 1** Demographic and Clinical Characteristics of the Patients Included in the Study

Variables	Total	Controls	Hypertension	Glaucoma	p-value
	N=97 161 eyes	N=38 62 eyes	N=31 53 eyes	N=28 46 eyes	
Gender, N (%)					
Females	56 (57.73)	21 (55.26)	15 (48.39)	20 (71.43)	0.1867*
Males	41 (42.27)	17 (44.74)	16 (51.61)	8 (28.57)	
Systemic hypertension, N (%)					
No	76 (78.35)	29 (76.32)	27 (87.10)	20 (71.43)	0.3195*
Yes	21 (21.65)	9 (23.68)	4 (12.90)	8 (28.57)	
Age, mean (SD)	58.73 (7.20)	57.10 (6.76)	60.00 (7.06)	59.54 (7.75)	0.1979^
Axial length, mean (SD)	23.70 (1.04)	23.73 (1.11)	23.44 (0.85)	23.95 (1.10)	0.9760°
Intraocular pressure (morning), mean (SD)	16.75 (3.32)	15.89 (2.51)	18.83 (3.67)	15.52 (3.13)	<0.0001°
Intraocular pressure (evening), mean (SD)	15.28 (3.15)	14.68 (2.32)	17.30 (3.23)	13.75 (2.86)	<0.0001°
Daily change in intraocular pressure, mean (SD)	1.47 (2.27)	1.21 (1.83)	1.53 (2.69)	1.76 (2.28)	0.5903°
Scan Quality (morning), median (IQR)	9 (8–9)	9 (8–9)	9 (8–9)	8 (8–9)	0.4203§
Scan Quality (evening), median (IQR)	9 (8–9)	9 (8–9)	9 (8–9)	8 (8–9)	0.4203§
BCVA, mean (SD)	0.98 (0.07)	1.00 (0.02)	0.99 (0.04)	0.96 (0.11)	0.1485°
Refraction, mean (SD)	0.00 (0.90)	0.06 (0.76)	0.00 (0.89)	−0.09 (1.07)	0.423°

Notes: \*P-values from  $\chi^2$  test. ^p-values obtained from ANOVA test. °p-values obtained from repeated measure ANOVA. §Wilcoxon sum rank test.

Abbreviation: IQR, interquartile range.

**Table 2** Means and Corresponding Standard Deviations (SD) of All Ocular Parameters Measured in the Morning and in the Evening with Respective Meand Daily Changes in the Three Groups of Patients. False Discovery Rate (FDR) Adjusted P-value Of the ANOVA Test are Shown. The Mean Measurements of All the Parameters Investigated Were Statistically Different in the Three Groups in the Morning and in the Evening. Mean Daily Changes Were Minimal and Not Statistically Significant

	Morning				Afternoon				Daily change			
	Control eyes		FDR adjusted p-value		Control eyes		FDR adjusted p-value		Control eyes		FDR adjusted p-value	
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
<b>Vascular density</b>												
WVD	49.76 (2.58)	49.06 (2.6)	42.2 (6.22)	<0.0001	49.65 (2.17)	48.72 (2.41)	42.45 (6.49)	<0.0001	0.12 (2.14)	0.34 (2.38)	-0.25 (1.99)	0.8324
ONH-VD	52.63 (4.27)	51.25 (4.54)	48.39 (7.21)	0.00775	52.48 (5.43)	51.55 (4.58)	48.93 (5.99)	0.01338	0.17 (3.88)	-0.31 (3.93)	-0.54 (4.56)	0.8324
PPVD	51.91 (3.21)	51.02 (2.46)	43.65 (7.45)	<0.0001	51.72 (2.73)	50.92 (2.72)	43.36 (9.56)	<0.0001	0.2 (2.37)	0.1 (2.37)	0.29 (6.34)	0.8335
PPHnfVD	51.75 (3.34)	50.79 (2.5)	43.29 (7.95)	<0.0001	51.59 (2.98)	50.63 (3.02)	43.77 (8.35)	<0.0001	0.17 (2.45)	0.16 (2.63)	-0.47 (2.46)	0.5810
PPHSup-VD	52.04 (3.43)	51.24 (2.76)	43.98 (7.68)	<0.0001	51.81 (2.87)	51.18 (2.84)	44.66 (8.46)	<0.0001	0.23 (2.62)	0.06 (2.4)	-0.68 (2.33)	0.5810
PPNI-VD	47.76 (4.93)	46.96 (4)	40.1 (8.23)	<0.0001	47.65 (4.76)	46.71 (4.91)	40.45 (8.11)	<0.0001	0.11 (3.12)	0.25 (3.75)	-0.35 (2.88)	0.8335
PPNS-VD	49.04 (4.32)	48.8 (3.67)	41.16 (7.5)	<0.0001	48.97 (3.98)	48.99 (3.98)	42.27 (8.4)	<0.0001	0.07 (2.73)	-0.19 (2.74)	-1.12 (2.45)	0.5468
PPIN-VD	50.67 (5.63)	49.11 (4.75)	40.12 (10.36)	<0.0001	50.49 (5.67)	49.86 (4.46)	40.53 (11.08)	<0.0001	0.17 (3.79)	-0.75 (3.42)	-0.41 (3.56)	0.5810
PPSN-VD	48.71 (5.07)	47.63 (4.43)	40.21 (8.63)	<0.0001	48.04 (4.46)	47.83 (4.72)	40.9 (9.43)	<0.0001	0.68 (3.74)	-0.2 (3.67)	-0.68 (3.28)	0.5810
PPIT-VD	58.36 (3.9)	56.72 (3.94)	47.98 (12.19)	<0.0001	58.29 (3.51)	57 (3.69)	48.06 (13.62)	<0.0001	0.07 (3.41)	-0.28 (3.36)	-0.07 (3.83)	0.8335
PPST-VD	55.61 (4.36)	54.52 (4.34)	45.53 (12.5)	<0.0001	55.42 (4.05)	54.22 (4.56)	45.62 (13.21)	<0.0001	0.19 (4.05)	0.31 (3.29)	-0.09 (4.59)	0.8324
PPTI-VD	52.04 (3.83)	52.01 (3.76)	46.59 (7.18)	<0.0001	51.79 (3.45)	50.58 (4.92)	47.7 (6.72)	0.00586	0.33 (2.86)	1.42 (4.49)	-1.1 (2.95)	0.0150
PPTS-VD	55.86 (3.79)	54.82 (3.65)	50.15 (8.25)	0.00016	56.17 (3.71)	54.38 (4.59)	50.24 (8.7)	0.00033	-0.2 (3.49)	0.44 (3.74)	-0.09 (3.1)	0.8335
<b>GCC</b>												
W-GCC	95.15 (5.07)	94.43 (5.82)	79.76 (12.66)	<0.0001	94.98 (5.69)	93.77 (5.78)	79.91 (12.8)	<0.0001	0.16 (2.19)	0.66 (2.72)	-0.15 (1.44)	0.5810
HInf-GCC	95.79 (5.07)	95.49 (6.3)	80.59 (13.28)	<0.0001	95.53 (5.26)	94.6 (5.96)	80.35 (13.57)	<0.0001	0.26 (2.09)	0.89 (3.27)	0.24 (3.02)	0.8324
HSup-GCC	94.61 (5.5)	93.25 (5.91)	79.13 (14.22)	<0.0001	94.58 (5.67)	93 (5.94)	79.15 (14.28)	<0.0001	0.03 (1.84)	0.25 (2.35)	-0.02 (1.29)	0.8324
<b>RNFL</b>												
ONH-RNFL	97.48 (7.19)	94.4 (8.29)	76.07 (15.95)	<0.0001	97.24 (7.28)	93.81 (8.33)	76.3 (15.13)	<0.0001	0.24 (1.77)	0.58 (2.11)	-0.24 (3.47)	0.9052
HInf-RNFL	96.1 (8.01)	93.43 (8.97)	74.13 (15.43)	<0.0001	96.11 (7.75)	92.98 (9.2)	73.83 (15.16)	<0.0001	-0.02 (2.52)	0.45 (2.21)	0.3 (2.23)	0.8335
HSup-RNFL	98.73 (7.58)	95.43 (8.99)	77.93 (18.08)	<0.0001	98.44 (7.88)	94.75 (8.82)	78.89 (17.31)	<0.0001	0.29 (2.54)	0.68 (3.40)	-0.96 (5.8)	0.8324
IN-RNFL	109.1 (19.47)	106.57 (16.02)	81.65 (21.94)	<0.0001	109.06 (18.61)	105.15 (16.08)	82.04 (22.42)	<0.0001	0.03 (5.73)	1.42 (6.66)	-0.39 (6.75)	0.8324
IT-RNFL	135.73 (13.61)	130.23 (16.56)	100.35 (26.12)	<0.0001	135.53 (11.95)	129.57 (16.47)	98.61 (26.66)	<0.0001	0.19 (5.49)	0.66 (7.08)	1.74 (6.62)	0.8324
NL-RNFL	72.63 (11.56)	70.04 (10.55)	58.65 (12.46)	<0.0001	72.68 (11.7)	67.96 (16.28)	60.39 (13.22)	0.00349	-0.05 (3.6)	2.08 (12.29)	-1.74 (6.81)	0.5468
SN-RNFL	100.58 (13.41)	102.25 (15.01)	83.61 (23.73)	0.00089	99.65 (12.95)	102.11 (14.32)	84.3 (22.66)	0.00129	0.94 (4.16)	0.13 (5.64)	-0.7 (4.95)	0.5810
ST-RNFL	132.92 (11.54)	124.28 (13.7)	101.48 (25.36)	<0.0001	132.53 (12.34)	122.77 (13.47)	102.46 (24.22)	<0.0001	0.39 (5.52)	1.51 (5.93)	-0.98 (7.45)	0.8324
TL-RNFL	66.9 (8.61)	65.62 (11.12)	55.98 (13.82)	<0.0001	67.24 (7.85)	67.11 (9.11)	54.78 (11.09)	<0.0001	-0.34 (4.9)	-1.49 (10.16)	1.2 (7.55)	0.8324
TU-RNFL	81.18 (9.96)	77.81 (12.15)	62.41 (18.87)	<0.0001	81.23 (9.75)	76.81 (13.83)	62.02 (18.61)	<0.0001	-0.05 (4.97)	1.00 (5.76)	0.39 (8.58)	0.8335
NU-RNFL	80.81 (12.39)	77.72 (11.77)	64.26 (14.36)	<0.0001	80.58 (12.38)	77.62 (12.77)	66.89 (16.12)	0.00011	0.23 (3.92)	0.09 (4.14)	-2.63 (11.49)	0.5810

**Table 3** The T-test Derived P-value Adjusted for Multiplicity Using the False Discovery Rate (FDR) Method. Daily Fluctuations Were Not Statistically Different in Any of the Parameters Investigated within the Groups and in the Total Sample. Only main parameters are indicated.

	Control eyes	Eyes with ocular hypertension	Eyes with glaucoma
	FDR adjusted p-value	FDR adjusted p-value	FDR adjusted p-value
<b>Vascular density</b>			
W-VD	0.7077	0.7907	0.9385
ONH-VD	0.7077	0.7907	0.9385
PP-VD	0.8377	0.7907	0.9385
PP Half Inf-VD	0.7077	0.8741	0.8146
PP Half Sup-VD	0.7077	0.8741	0.8146
<b>GCC</b>			
W-GCC	0.7077	0.7823	0.8146
Half Inf-GCC	0.8347	0.7823	0.9385
Half Sup-GCC	0.7077	0.8905	0.9385
<b>RNFL</b>			
ONH-RNFL	0.7077	0.8004	0.9385
Half Inf-RNFL	0.9415	0.7907	0.9385
Half Sup-RNFL	0.7077	0.8741	0.9385

## Discussion

This study provides insight into quantitative morphological and vascular-derived characteristics determined by means of OCTA imaging technology in a cohort of individuals homogeneous for age, gender, refractive status, visual acuity, systemic hypertension. Vessel density, GCC and RNFL proved to stay during the daytime, thus corroborating the clinical reliability of this technology in routine clinical practice.

Since eyes with glaucoma and with ocular hypertension were treated with topical medications, it is not surprising there were significant differences among groups in IOP measurements taken in the morning and in the evening (Table 1,  $P < 0.0001$ ), though there were no significant daily changes (Table 1,  $P = 0.59$ ).

Consistently with previous reports,<sup>10–15</sup> we found that eyes with glaucoma exhibited RNFL, GCC and VD reduction in every sector of the peripapillary area investigated. This reduction was statistically significant in the morning and the evening in all three groups including every single

characteristic (Table 2,  $P < 0.02$ ). Similarly, when comparing group to group, the means of all the parameters measured in the morning and in the evening differed significantly between eyes with glaucoma and eyes with ocular hypertension and controls except ONH-VD and NL-RNFL in the evening (Table 4,  $p < 0.008$ ). On the contrary, no significant differences were registered between control eyes and eyes affected by ocular hypertension ( $P > 0.17$ , Table 4). In fact, in the ocular hypertension group RNFL, GCC and VD were slightly reduced, but not statistically significantly. This finding is not concordant with several previous studies in which significant reductions in RNFL, GCC and VD were considered to precede glaucoma development.<sup>7,15–18</sup> Notably, we included eyes with ocular hypertension that had not alteration of the ONH and this may explain the discrepancy.

Vessel density is thought to be a reliable indicator of microcirculation status, and it has been postulated that vascular problems involving chronic impairment of ocular blood flow can lead to RNFL/GCC damage.<sup>19–21</sup> The increasing use of OCTA in clinical practice may clarify the role of ocular blood flow in the pathogenesis of several retinal diseases. Although several potential artefacts constitute a limitation of OCTA technology, its reproducibility is considered good and intra-session and inter-session coefficients ranging from 1.8% to 7.0% have been reported.<sup>22–24</sup> However, diurnal VD variations have not been thoroughly investigated to date, in eyes with or without glaucoma. Mansouri et al recently concluded that diurnal differences were relatively small and not statistically significant, although VD measurements of the whole peripapillary area, including the ONH and the peripapillary area were higher in the afternoon than in the morning in a sample of 86 eyes from 43 glaucoma patients.<sup>19</sup> Overall, these findings are consistent with the current study in which not only 46 eyes from 28 individuals with glaucoma, but also the 53 eyes from 31 patients affected by ocular hypertension, and the 62 eyes from 38 controls were analysed too. The diurnal changes detected were minimal in every area and in every group, and there were no statistically significant differences in this respect (Tables 2 and 3).

High-quality OCTA imaging is essential for interpreting and monitoring VD. Mansouri et al found that changes in scan quality and VD were significantly associated.<sup>19</sup> On the contrary, it is relevant that no statistical correlations were detected in our series, probably because of the high quality of scan acquisition (median 8–9, Table 1).

**Table 4** Post-hoc tukey's tests of the Group to Group Comparison. There Is a Statistically Significant Difference for Every Parameter Investigated between Eyes with Glaucoma and Control Eyes or with Ocular Hypertension, except for ONH-VD in the Evening. Comparisons between Eyes with Ocular Hypertension and Control Eyes Were Not Statistically Significant. Only main parameters are indicated.

Variable	Morning			Evening		
	Glaucoma vs Hypertension	Glaucoma vs Controls	Hypertension vs Controls	Glaucoma vs Hypertension	Glaucoma vs Controls	Hypertension vs Controls
	Tukey adjusted p-value	Tukey adjusted p-value	Tukey adjusted p-value	Tukey adjusted p-value	Tukey adjusted p-value	Tukey adjusted p-value
<b>Vascular density</b>						
W-VD	<0.0001	<0.0001	0.5536	<0.0001	<0.0001	0.5977
ONH-VD	0.0071	0.0059	0.6308	0.0821	0.0115	0.7432
PP-VD	<0.0001	<0.0001	0.4448	<0.0001	<0.0001	0.6998
PPHInf-VD	<0.0001	<0.0001	0.3844	<0.0001	<0.0001	0.6474
PPHSup-VD	<0.0001	<0.0001	0.6330	<0.0001	<0.0001	0.8565
<b>GCC</b>						
W-GCC	<0.0001	<0.0001	0.8826	<0.0001	<0.0001	0.8494
HInf-GCC	<0.0001	<0.0001	0.9138	<0.0001	<0.0001	0.8234
HSup-GCC	<0.0001	<0.0001	0.8623	<0.0001	<0.0001	0.8384
<b>RNFL</b>						
ONH-RNFL	<0.0001	<0.0001	0.2935	<0.0001	<0.0001	0.1945
HInf-RNFL	<0.0001	<0.0001	0.2295	<0.0001	<0.0001	0.1779
HSup-RNFL	<0.0001	<0.0001	0.5173	<0.0001	<0.0001	0.3609

The main interesting finding of the study is that all the parameters investigated were stable throughout the day in all three groups despite the fact that physiological variations in IOP, systemic pressure and choroidal blood flow were reportedly typical.<sup>25</sup> We can observe that in eyes with glaucoma the VD in most of the sectors tend to be higher in the evening, but this finding is not statistical significant. Moreover, it is known that the choroidal thickness changes during the day<sup>26,27</sup> and that choroid has autonomic nerve endings whereas the retina does not.<sup>28</sup> Consistently, in the study by Yanik et al, no daily fluctuations were found in macular VD<sup>28</sup> probably because the retinal circulation is not an end artery region like the choriocapillary, but is organized in superficial and deep vascular plexuses. In addition, given that VD is a validated value determined automatically by a device which calculates the total area investigated/the area occupied by vessels based on retina's en face imaging, the present study yielded some additional evidence that retinal vessels are influenced by an autoregulatory system in order to maintain stable vascularity that is independent of other factors.<sup>28,29</sup> Conversely, a number of previous studies

indicated abnormal ocular blood flow autoregulation in glaucoma using different technologies such as color/laser Doppler flowmetry.<sup>30,31</sup> However, it should be noted that OCTA can quantitatively assess vessel calibre volume in the area investigated, but not the amount of blood flow. Moreover, all patients with glaucoma or ocular hypertension in the present study were on IOP-lowering topical medications, known to normalize ocular blood flow.<sup>32</sup>

To the best of our knowledge there are few reports on diurnal fluctuations in GCC and RNFL thickness determined by spectral domain optical coherence tomography (SD-OCT). Using a different device, the Spectralis SD-OCT (Heidelberg Engineering, Heidelberg, Germany) Ashraf et al evidenced that in 21 healthy individuals RNFL thickness was significantly greater in the morning in nasal, inferior and inferonasal subfields, suggesting that changes were due to real diurnal variations rather than repeatability limitations of the device.<sup>33</sup> On the contrary, our findings suggest morphological stability of the RNFL and GCC thickness in the eyes with and without glaucoma during the day.

**Table 5** Regression Coefficients ( $\beta$ ) and Corresponding Standard Error (SE) with FDR Adjusted P-value Derived from Multivariate Model, Systemic Hypertension, Age, Axial Length and Daily Changes in IOP and Scan Quality Were Not Significantly Associated with Daily Changes in Any of the Parameter Investigated

	Systemic Hypertension		Age		Axial length		Daily changes in intraocular pressure		Scan Quality	
	$\beta$ (SE)	FDR adjusted p-value*	$\beta$ (SE)	FDR adjusted p-value*	$\beta$ (SE)	FDR adjusted p-value*	$\beta$ (SE)	FDR adjusted p-value*	$\beta$ (SE)	FDR adjusted p-value*
<b>Vascular density</b>										
W-VD	0.42 (0.42)	0.7912	0.04 (0.03)	0.5536	-0.33 (0.18)	0.6525	0.07 (0.08)	0.7112	-0.07 (0.26)	0.9169
ONH-VD	0.81 (0.77)	0.7912	0.03 (0.05)	0.7712	-0.95 (0.34)	0.1525	0.1 (0.14)	0.7112	0.75 (0.47)	0.6071
PP-VD	0.62 (0.62)	0.7912	-0.02 (0.04)	0.807	-0.35 (0.28)	0.6782	0.03 (0.12)	0.8978	0.04 (0.37)	0.9169
PP Half Inf-VD	0.21 (0.48)	0.7912	0.04 (0.03)	0.5536	-0.16 (0.22)	0.8679	0.05 (0.09)	0.7805	-0.33 (0.3)	0.9003
PP Half Sup-VD	0.58 (0.5)	0.7912	0.04 (0.03)	0.5536	-0.09 (0.22)	0.9211	0.12 (0.09)	0.7112	-0.07 (0.3)	0.9169
PPNI-VD	0.58 (0.69)	0.7912	0.02 (0.04)	0.807	-0.37 (0.3)	0.6782	0.18 (0.12)	0.7112	0.09 (0.4)	0.9169
PPNS-VD	-0.25 (0.53)	0.7912	0.05 (0.03)	0.5536	-0.19 (0.23)	0.8679	0.07 (0.09)	0.7112	0.53 (0.32)	0.6071
PPIN-VD	-0.04 (0.68)	0.958	0.06 (0.04)	0.5536	-0.02 (0.3)	0.9558	-0.08 (0.12)	0.7112	-0.27 (0.42)	0.9169
PPSN-VD	1.24 (0.78)	0.7912	0.02 (0.05)	0.807	-0.06 (0.33)	0.9231	0.08 (0.13)	0.762	-0.43 (0.44)	0.9003
PPT-VD	-0.34 (0.64)	0.7912	0.01 (0.04)	0.807	-0.03 (0.28)	0.9558	-0.04 (0.12)	0.8596	0.15 (0.4)	0.9169
PPST-VD	1.39 (0.76)	0.7912	0.04 (0.05)	0.7712	0.23 (0.33)	0.8679	0.27 (0.14)	0.6207	0.1 (0.47)	0.9169
PPTI-VD	0.25 (0.65)	0.7912	0.04 (0.04)	0.7116	-0.39 (0.29)	0.6782	0.14 (0.12)	0.7112	-1.1 (0.4)	0.1788
PPTS-VD	0.38 (0.69)	0.7912	0.05 (0.04)	0.5661	-0.06 (0.31)	0.9231	0.08 (0.13)	0.7112	-0.14 (0.41)	0.9169
<b>GCC</b>										
W-GCC	-0.15 (0.39)	0.7912	0.04 (0.03)	0.5536	0.26 (0.17)	0.6525	-0.09 (0.07)	0.7112	-0.21 (0.24)	0.9169
Half Inf-GCC	-0.34 (0.5)	0.7912	0.01 (0.03)	0.807	0.3 (0.21)	0.6782	-0.1 (0.09)	0.7112	0.09 (0.31)	0.9169
Half Sup-GCC	0.24 (0.3)	0.7912	0.04 (0.02)	0.5536	0.21 (0.13)	0.6525	0.05 (0.05)	0.7112	-0.33 (0.19)	0.6071
<b>RNFL</b>										
ONH-RNFL	-0.51 (0.46)	0.7912	0.04 (0.03)	0.5536	-0.07 (0.21)	0.9211	-0.07 (0.08)	0.7112	-0.08 (0.26)	0.9169
Half Inf-RNFL	-0.15 (0.47)	0.8177	0.01 (0.03)	0.807	-0.15 (0.21)	0.8679	0.01 (0.08)	0.931	0.29 (0.28)	0.9003
Half Sup-RNFL	-0.44 (0.66)	0.7912	0.05 (0.04)	0.5536	0.11 (0.3)	0.9211	0 (0.12)	0.9801	-0.44 (0.37)	0.9003
IN-RNFL	1.43 (1.23)	0.7912	0.09 (0.08)	0.5588	-0.56 (0.54)	0.8346	-0.18 (0.23)	0.7112	0.09 (0.75)	0.9169
IT-RNFL	-1.6 (1.23)	0.7912	0 (0.08)	0.9682	-0.25 (0.54)	0.9211	0.09 (0.23)	0.8363	-0.11 (0.75)	0.9169
NIL-RNFL	2.28 (1.58)	0.7912	0.06 (0.1)	0.807	0.46 (0.7)	0.8679	-0.51 (0.29)	0.6207	0.33 (0.97)	0.9169
SN-RNFL	-0.38 (0.95)	0.7912	-0.01 (0.06)	0.9371	-0.68 (0.42)	0.6525	-0.18 (0.17)	0.7112	-1.33 (0.57)	0.2908
ST-RNFL	-0.66 (1.29)	0.7912	0.1 (0.08)	0.5536	0.27 (0.57)	0.9211	-0.01 (0.22)	0.9801	-0.15 (0.72)	0.9169
TL-RNFL	-0.83 (1.2)	0.7912	-0.08 (0.08)	0.5588	-0.18 (0.55)	0.9211	0.48 (0.23)	0.6207	-0.72 (0.72)	0.9003
TU-RNFL	-0.31 (1.22)	0.831	0.15 (0.08)	0.5536	0.12 (0.55)	0.9231	0.25 (0.22)	0.7112	-0.16 (0.73)	0.9169
NIU-RNFL	0.75 (1.02)	0.7912	0.02 (0.06)	0.807	0.35 (0.47)	0.8679	-0.33 (0.19)	0.6207	0.48 (0.6)	0.9169

The regression coefficient derived from the multivariate model investigated the roles of systemic hypertension, axial length and IOP changes. Interestingly, none of these factors were shown to play a significant role (Table 5). Although not statistically significant, increased age corresponded with small increases in mean daily variation in almost all of the parameters investigated, suggesting that aging may affect the vascular autoregulatory system of the eye.

The current study had some limitations, including the intrinsic limit of OCTA technology and the lack of multiple intra-session acquisitions. However, it is unique in that it compared diurnal fluctuations in OCTA-determined parameters and in more commonly used RNFL and GCC thickness, in the same eye, with the same device in different groups of patients. None of the parameters investigated exhibited significant daily variation, which was independent of age, IOP, systemic hypertension and axial length. Overall, all the measurements were stable during the day, thus corroborating the clinical relevance of OCTA regardless the time of acquisition.

## Conclusion

On optical coherence tomography angiography we did not find any diurnal fluctuations in peripapillary vessel density and thickness of ganglion cell complex and retinal fiber layer, when we compared healthy eyes with eyes of patients with ocular hypertension and glaucoma.

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## Disclosure

The authors report no conflicts of interest in this work.

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