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Role of multifocal electroretinogram in assessment of early retinal dysfunction in hypertensive patients

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Ayat Allah Farouk[1], Reem ElHadidy[1], Eman Attia Abd ElSalam[1], Rasha Zedan[2] and Radwa Azmy[1]

Abstract

Purpose: To investigate localized retinal dysfunction in hypertensive patients using multifocal electroretinogram (mfERG) and to assess its sensitivity as an early predictor for the development of retinopathy in hypertensive patients.
Methods: Ninety-eight eyes were included in this case-control study. Twenty-eight eyes of healthy subjects served as a control group (group I). Seventy eyes belonged to patients with systemic hypertension assigned into two groups; group II including 39 eyes of hypertensive patients with normal fundus and group III including 31 eyes of patients with signs of hypertensive retinopathy. All participants were subjected to complete ophthalmic and electrophysiological examination using mfERG. N1 and P1 wave amplitudes and implicit times from the central hexagon and four concentric rings across the visual field were analyzed.
Results: mfERG amplitudes were significantly reduced in hypertensive group with retinopathy than in controls. N1 amplitude was significantly reduced in the most eccentric ring in eyes of hypertensive patients with normal fundus.
Conclusion: mfERG is a sensitive objective tool for assessment of retinal dysfunction in hypertensive patients. mfERG amplitude is a promising predictor for early development of retinopathy in systemic hypertension.

Keywords

Systemic hypertension, hypertensive retinopathy, multifocal electroretinogram, mfERG

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Introduction

Hypertension is a chief cause of disability and mortality all over the world.[1] It is the most common chronic medical condition in Egypt, with a prevalence rate of 26.3% among the adult population.[2] Hypertension has profound effects on the structure and function of the eye, including the retina, resulting in hypertensive retinopathy.[3] Hypertensive retinopathy is the second most common cause of retinopathy following diabetes, probably caused by retinal ischemia as a consequence of atherosclerotic irreversible changes of systemic hypertension.[4]

Functional abnormalities of the retina may precede the clinical signs of retinopathy in systemic hypertension. Hypertensive retinopathy signs could be a predictor of incident stroke and cardiovascular mortality. Utilizing objective tests for early detection of retinal dysfunction in hypertensive patients before the appearance of signs in fundoscopy would be of great value.[3]

Retinal electrophysiology, namely, mfERG offers an objective noninvasive and reliable method to measure retinal function.[5] It allows simultaneous recording of local ERG responses from different parts of the retina.[6] Non-uniform functional retinal abnormalities is difficult to detect by

[1]Clinical Neurophysiology Unit, Kasr Al Ainy Hospital, Cairo University, Cairo, Egypt
[2]Department of Opthalmology, Kasr Al Ainy Hospital, Cairo University, Cairo, Egypt

Corresponding author:
Radwa Azmy, Clinical Neurophysiology Unit, Kasr Al Aini, Cairo University Hospitals, 11562 Cairo, Egypt.
Email: radwa_azmy@yahoo.com

full-field ERG as it represents summated responses recorded from the entire retina and needs the disease to reach advanced changes to be affected.[7] The photoreceptors and the inner retina including bipolar cells, are the primary generators of N1–P1 of mfERG. The fovea being an avascular zone is highly sensitive to inadequate blood perfusion in the choroidal circulation and that can be tested via N1 wave.[4] Ischemic damage of the inner nuclear retinal layer where the cell bodies of the bipolar cells lie can be tested via P1 wave.[8]

By reviewing literature, only few researchers have investigated retinal electrophysiology in hypertensive retinopathy using mfERG. To the best of our knowledge, no previous study has reported the role of mfERG examination in prediction of early development of retinopathy in hypertensive patients

Subjects and methods

This is a case control study carried out on hypertensive patients recruited from the Internal medicine outpatient clinic, at Kasr Alainy Hospital, Cairo University. Neurophysiological and ophthalmological assessments were carried out at Neurophysiology Unit of Kasr Alainy Hospital & Ophthalmology outpatient Clinic of Kasr Alainy Hospital.

Ninety-eight eyes were included in this study as per sample size estimation. Twenty-eight eyes were of normotensive healthy subjects whose blood pressure was $<130/80$ and no prior antihypertensive medication and 70 eyes were of essential hypertensive patients on antihypertensive medications for more than 1 year. Sample size was calculated using G*Power program (University of Düsseldorf, Düsseldorf, Germany).

Informed consent was obtained from all subjects involved in the study. Procedures followed the declaration of Heliniski and the protocol was approved by the ethical committee, Cairo University.

All participants were subjected to clinical and ophthalmological examination. Fundoscopy and slit-lamp biomicroscopy of anterior and posterior segment were done by a single ophthalmologist. Random blood sugar was measured to exclude diabetic patients. Blood pressure measurement was done twice for all the patients and controls. Antihypertensive medications were reported.

Patients with essential hypertension on oral antihypertensive medications were included in this study. Patients with diabetes, renal disease, significant anterior segment diseases, for example, cataract or glaucoma, significant posterior segment diseases, or surgery were excluded from the study. Hypertensive retinopathy was evaluated according to a simple three-grade classification system suggested by Wong and McIntosh in 2005.[9] The eyes of hypertensive patients were further assigned into two groups based on fundus findings.

mfERG was carried out, according to ISCEV standards (International Society for Clinical Electrophysiology of Vision) using the METROVISION scan version 8000F (Metrovision, Francais). Researchers were blinded to fundus findings when performing retinal electrophysiological studies. The stimulus consisted of 61 hexagons, covering 25° to 30° of visual field to either side of fixation, at a frequency of 15 Hz with a stimulation presentation of 33 ms using an LCD (liquid crystal display) panel with LED (light emitting diode) backlight, on a 20-inch monitor at a distance of 33 cm (stimulated field of $\pm 30°$ horizontally and $\pm 24°$ vertically). Signals were amplified with a gain of 100 μV and filtered with a band-pass filter (5–300 Hz).

Areas of the hexagons increase eccentrically to overcome cone density difference across the retina. Each session lasted 6 minutes, broken into 45-second segments, eight trials were recorded in total. The noise level was kept less than 5 μV.

A map of amplitudes or implicit times of N1, P1, N2, and P2 wave peaks was then displayed. Values were provided in nV/deg^2. The 3D color map was obtained by direct interpolation between the measuring points. mfERG evaluates the local retinal functions from the fovea to the peripheral 30°, dividing this area into up to five retinal zones (central hexagon and four rings). Central hexagon (fovea) "F": from 0° to 2° of eccentricity relative to the fixation, R1: from 2° to 5°, R2: from 5° to 10°, R3: from 10° to 15°, and R4: over 15°.

For each ring, amplitude and the peak implicit time of P1 and N1 components were calculated. Average responses were calculated for the central hexagon and for four retinal rings. Only the first order kernel responses were analyzed.

The data was analyzed using Microsoft Excel 2010 (Microsoft Corporation, New York, USA) and IBM SPSS version 21.0 (IBM Co., USA). Chi-square test of normality and normal distribution of the data from the rings and fovea was assumed. Comparison was done using One-way ANOVA with Bonferroni correction for post-hoc analysis as the data was normally distributed. Categorical data was described with chi-square. Correlations were done by Pearson correlation coefficient (r). P-value $\leqslant 0.05$ will be considered statistically significant. Cat-sensitivity test was done, using our laboratory normative data.

Results

This is a case-control study, conducted on 98 eyes, divided into three groups, control group (group I) including 28 eyes and study group consisting of 70 eyes of essential hypertensive patients who were further subdivided according to fundus findings into group II including 39 eyes of hypertensive patients with normal fundus, and group III including 31 eyes of patients with signs of hypertensive retinopathy. All patients in groups II and III were known to be controlled on oral antihypertensive medications. Demographic data of the three study groups is shown in Table 1. A sample of mfERG test in one of patients included in the present study is shown in Figure 3.

Table 1. Demographic characteristics in the study groups.

	Group I Normal control ($n=28$)	Group II Hypertensive patients with normal fundus ($n=39$)	Group III Hypertensive patients with signs of retinopathy ($n=31$)	p value
Age (year, mean ± SD)	46.0 ± 11	47.0 ± 8	51.0 ± 8	0.083*
Eyes in study (OD/OS)	15/13	19/20	16/15	0.812**
Eyes per gender (M/F; %)	15/13 (53.60%/46.40%)	18/21 (46.20%/53.80%)	8/23 (25.80%/74.20%)	0.67**
Duration of hypertension (years, mean ± SD)	N/A	4.1 ± 4	10.2 ± 5.4	<0.001***

M: male; F: female; SD: standard deviation; OD: right; OS: left; N/A: not applicable.
*One-way ANOVA; **Chi square test; ***Mann–Whitney test.

Table 2. Average P1 amplitudes (nV/deg^2) in the three study groups.

	Group I		Group II		Group III		p value
	Mean	SD	Mean	SD	Mean	SD	
P1 amp "F"CH	928.9	341.1	861.8	323.4	759.5	412.3	0.191
P1 amp R1	485.8	163.3	394.1	161.8	406.6	184.8	0.078
P1 amp R2	397.1	173.4	357.9	126.9	335.2	147.6	0.274
P1 amp R3	424.4a	150.3	330.1	115.0	331.5a	156.9	0.013
P1 amp R4	443.1a–b	169.0	339.8a	108.3	322.6b	158.1	0.003

SD: standard deviation.
$p \leq 0.05$ is considered statistically significant and analysis done by ANOVA test and post hoc analysis.
Similar letters in the same row are statistically significant by post hoc analysis; a-b (a and b).

mfERG findings

Mean amplitudes of P1 in the three groups in the central hexagon "F" and four concentric rings are shown in Table 2 and Figure 1. There was a statistically significant difference between the P1 amplitudes of the three study groups ($p \leq 0.05$), noted in R3 and R4 (the most eccentric rings). In post hoc analysis, R3 showed a statistically significant amplitude drop in group III compared to group I ($p=0.013$), whereas R4 showed a statistically significant drop in group III compared to group I, and group III compared to II ($p=0.003$). There was no statistically significant difference between mean implicit times of P1 in the fovea and four concentric rings of the three groups ($p>0.05$, One-Way ANOVA) Table 3.

Mean amplitudes of N1 in the three groups are shown in Table 4 and Figure 2. Only the central hexagon "F" and R4 showed a statistically significant difference between the N1 amplitudes of the three study groups ($p \leq 0.05$, One-Way ANOVA). In post hoc analysis, "F" showed a statistically significant differences between the groups I and III ($p=0.018$), whereas R4 showed a statistically significant difference between groups I and II and groups I and III ($p=0.001$).

The Mean implicit times of N1 of the three groups in the central hexagon and four concentric rings are shown in Table 5. There was a statistically significant prolongation of the implicit time in group III compared to group I noted in R2 and to group II in R4.Correlations between the age and average N1 and P1 amplitudes and implicit times in groups II and III are shown in Tables 6 and 7.

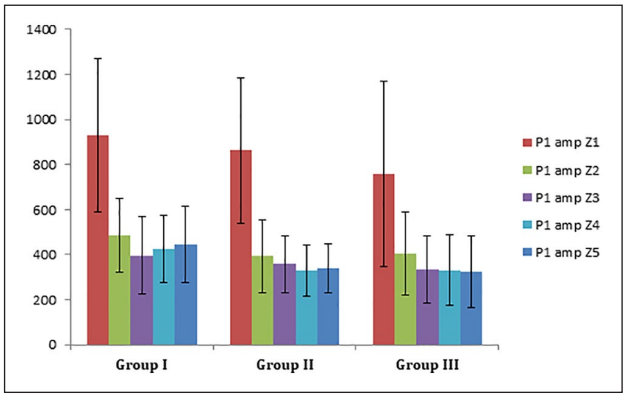

Figure 1. Average P1 amplitudes (nV/deg^2) in the three study groups (Z refers to rings R).

In group III, 22 of the 31 examined eyes showed amplitude reduction that achieved 70.9% sensitivity for diagnosis of hypertensive retinopathy. Of mfERG variables, a low amplitude of R4 and the central hexagon, followed by R3 was more sensitive than the other rings. In group II, 27 of the 39 examined eyes amplitude reduction that achieved 69.2% for prediction of retinopathy development in hypertensive patients.

Discussion

Hypertension is a major health problem worldwide that has profound effects on the structure and function of the eye

Table 3. Average P1 implicit times (msec) in the three study groups and ANOVA tests results and *p*-value.

	Group I		Group II		Group III		*p* value
	Mean	SD	Mean	SD	Mean	SD	
P1 lat CH"F"	53.4	4.2	54.4	6.6	51.9	11.3	0.424
P1 lat R1	48.9	4.6	48.9	2.5	48.9	9.3	1.000
P1 lat R2	61.7	5.7	47.6	3.6	48.7	4.8	0.324
P1 lat R3	47.6	4.5	46.8	2.5	46.4	9.2	0.745
P1 lat R4	48.0	4.4	47.5	3.6	49.8	5.1	0.092

Table 4. Average N1 amplitudes (nV/deg^2) in the three study groups.

	Group I		Group II		Group III		*p* value
	Mean	SD	Mean	SD	Mean	SD	
N1 amp CH"F"	568.8a	173.2	448.9	240.9	400.3a	258.2	0.018
N1 amp R1	226.5	62.5	221.7	100.9	224.4	93.9	0.976
N1 amp R2	215.6	92.2	192.7	89.1	169.7	68.9	0.118
N1 amp R3	219.4	111.6	168.7	97.3	169.7	64.8	0.059
N1 amp R4	222.9a–b	100.5	162.8a	50.4	149.2b	81.3	0.001

Similar letters in the same row are statistically significant by post hoc analysis; a–b (a and b).
P value mentioned is for post hoc analysis of the same row values, no separate p values by post hoc analysis.

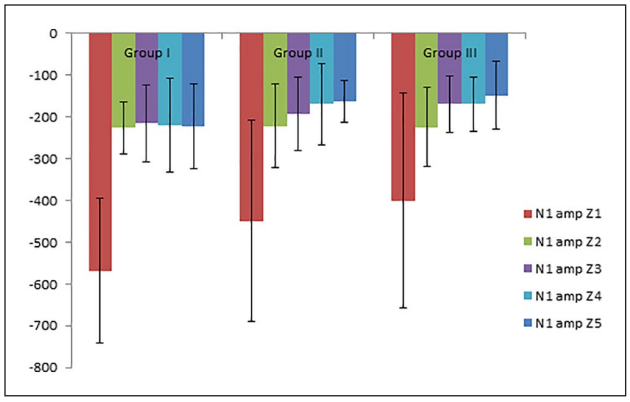

Figure 2. Average N1 amplitudes (nV/deg^2) in the three study groups (Z refers to rings R).

causing retinopathy, choroidopathy, and papilledema.[3,10] Prediction of hypertensive retinopathy is more valuable than its recognition.[8,11] Multifocal ERG recordings allows testing the retinal function across the central 40° to 50°.[7] Authors suggested that the abnormalities in retinal electrophysiological studies usually precede fundus signs of retinopathy as mfERG is affected early by the neurodegenerative process.[8,12] Researchers suggest that mfERG can detect alteration of retinal physiology inspite of otherwise normal anatomical and functional investigations.[6,13]

In the present study, the authors investigated mfERG in 98 eyes. According to ISCEV recommendations, each laboratory must report its own normative data due to variation in equipments and parameters rendering the use of data from other sources inappropriate; which was fulfilled in the present study by comparing the values of the patients' group to that of the control group; in addition to normal variation among populations due to several factors including age, ethnicity and size of the pupil.[7,13]

Analysis of mfERG was based upon concentric ring averages; central hexagon (CH) and four concentric rings (R1, R2, R3, and R4); across the retinal field. This analysis is considered more relevant than quadrant or single hexagon analysis in detection of early localized retinal dysfunction. In addition, it substantially reduces the noise contribution to the signal.[14] Bearse and Ozawa in 2014, compared waveform amplitudes for each hexagon to control values, while Gränse et al.[15] compared amplitudes of waveforms averaged over multiple hexagons arranged in rings.

Regarding group II eyes, both N1 and P1 amplitudes were significantly reduced compared to controls (group I) in ring "R4". The N1 peak time was prolonged than controls in the same ring as well. Group III eyes showed a significant P1 amplitude drop in the most eccentric rings R3 and R4, and significant N1amplitude drop in the central hexagon and R4. Significant prolongation of N1 implicit time was noted in R2 and R4 with no significant changes in P1 implicit time. These findings come in accordance with Gundogan et al.,[4] who investigated retinal dysfunction in patients with mild to moderate essential hypertension with signs of retinopathy. They reported P1 and N1 amplitude reduction with no significant difference in the terms of N1 and P1 peak time. They demonstrated that amplitude measures are more sensitive compared to implicit time changes in detecting retinal dysfunction in hypertensive retinopathy signs prior to initiation of antihypertensive medications. However, the distribution of

Table 5. Average N1 implicit times (msec) in the three study groups.

	Group I		Group II		Group III		*p* value
	Mean	SD	Mean	SD	Mean	SD	
N1 lat CH"F"	29.7	3.6	29.0	4.5	31.0	6.0	0.211
N1 lat R1	28.7	5.1	29.5	3.4	30.2	3.9	0.373
N1 lat R2	27.9a	4.1	28.5	2.2	30.0a	3.3	0.034
N1 lat R3	27.9	2.8	28.4	2.6	28.7	6.2	0.72
N1 lat R4	29.4	4.0	27.9a	5.3	31.2a	4.2	0.018

Table 6. Correlation between the age and the average N1 and P1 amplitudes (nV/deg^2) and implicit times (msec) in group II.

	N1 wave				P1 wave			
	Amplitude		Implicit time		Amplitude		Implicit time	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
CH	0.139	0.399	0.217	0.186	−0.316	0.050	0.055	0.738
R1	0.462**	0.003	0.123	0.457	−0.322*	0.046	0.369*	0.021
R2	0.158	0.338	0.230	0.158	−0.145	0.377	0.166	0.021
R3	0.170	0.302	0.241	0.140	−0.369*	0.021	0.292	0.072
R4	0.028	0.866	0.020	0.905	−0.351*	0.028	0.196	0.231

r: correlation coefficient.
*Significant (>0.3), **highly significant (0.3–0.5).

Table 7. Correlation between the age and the average amplitude (nV/deg^2) and implicit time (msec) in group III.

	N1 wave				P1 wave			
	Amplitude		Implicit time		Amplitude		Implicit time	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
CH	0.129	0.489	−0.026	0.891	0.025	0.893	−0.042	0.821
R1	−0.193	0.297	0.296	0.105	0.089	0.636	0.014	0.941
R2	−0.020	0.915	−0.057	0.761	−0.012	0.951	−0.014	0.942
R3	−0.261	0.156	0.545**	0.002	0.111	0.553	0.526**	0.002
R4	−0.361*	0.046	0.232	0.210	0.009	0.963	−0.119	0.525

r: correlation coefficient.
*Significant (>0.3), **highly significant (0.3–0.5).

mfERG amplitude abnormalities has shown considerable variation between the two studies. Similar results were noted in R3 P1 amplitude. This variation could be due to difference in subjects included as patients in the present study were on antihypertensive medication.

In the present study, reduction of amplitude was noted in the most eccentric two rings (R3 and R4). Researchers suggested that sustained vasospasm in hypertension affects the periphery of the macula.[16,17] Ibrahim et al.[18] stated that the number of perifoveal vessels decreased significantly in patients with hypertension. In addition, direct microvascular damage of high blood pressure could impair the blood flow to the optic nerve and lead to breakdown of the blood retinal barrier resulting in hemorrhage and exudate with subsequent ischemia of the nerve fiber layer.[3]

Moreover, the duration of hypertension had no correlation with either the amplitude or peak time of mfERG in group II in line with Gundogan et al.[4] On the other hand, group II showed significant positive correlation with peak times of N1 and P1. The contradictory findings may be also due to the diversity among the subjects recruited; each study included different groups of patients with different stages of hypertension, hypertensive retinopathy, disease duration, on treatment or not, and blood pressure control.

Furthermore, the present study correlated the age of the subjects with N1 and P1 amplitudes and implicit times. We found that the age of the patients affected the implicit times rather than amplitudes of mfERG. As the age advances, the peak time of N1 and P1 delays in controls and both hypertensive groups. This positive correlation

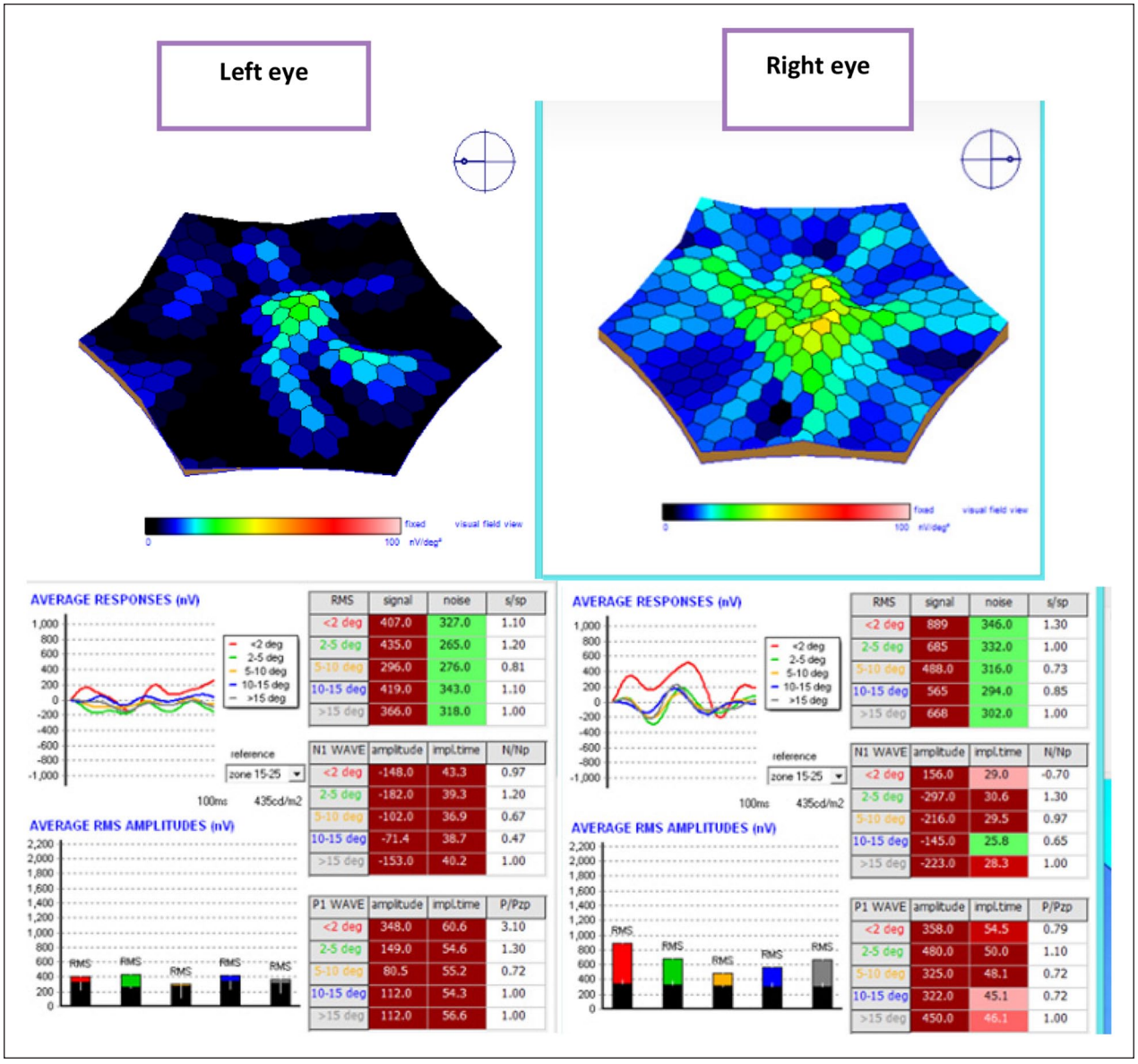

Figure 3. A 3D map and average amplitude and implicit time of each ring and central hexagon of both eyes of hypertensive patient with normal fundus. The average N1 and P1 waves amplitudes of the left eye are significantly reduced compared to the right eye, especially the most eccentric ring "R4."

was statistically significant in "R3" in group III and in R1 in group II. There was a negative correlation between the age of patients and wave amplitude in hypertensive groups only, significant in R4 in both hypertensive groups which would rather be explained by hypertension than by aging. This runs in context with Tzekov et al.[14] who reported that the age-related localized changes in the retina were not observable in the ring averages of mfERG. Curcio et al.[19] stated that the changes in cone density showed no consistent relationship to age, as the total number of foveal cones was remarkably stable throughout a wide age span, in contrast the rods density that declined by aging. The effect of age on mfERG peak time and amplitude as seen from the above results could be the result of either a combined effect of both aging and hypertension or a delay in diagnosis that prolonged the actual duration of the disease.

From the above, we postulate that the macular area more likely to develop hypertensive retinopathy is "R4" which represents the most eccentric ring (>15°). This could be attributed to vasoconstriction of retinal arterioles in response to hypertension, resulting in disturbance of autoregulation mechanism of the retinal circulation.[16] In hypertensive patients, with or without retinopathy signs, mfERG amplitude loss was more prominent than the prolongation of implicit time. These changes were evident in the foveal and perifoveal areas. Sensitivity of mfERG amplitude in the

present study, was found to be 90.3% in "R4" and 77.4% in the central hexagon and could predict the development of retinopathy in "R4" with 84.6% sensitivity.

In conclusion, mfERG is a promising predictor for the development of hypertensive retinopathy. N1 and P1 waves mfERG amplitude, especially "R4"is recommended to predict early signs of hypertensive retinopathy. The current study sheds light on the value of mfERG in early diagnosis of retinal dysfunction in hypertensive patients before detecting it by fundus examination which can help prevent other atherosclerotic complications of hypertension.

Declaration of conflicting interests

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ORCID iD

Radwa Azmy https://orcid.org/0000-0003-3403-6119

References

1. Angeli F, Reboldi G, Trapasso M, et al. European and US guidelines for arterial hypertension: similarities and differences. *Eur J Intern Med* 2019; 63: 3–8.
2. Hasan DH, Emeash AB, Mustafa S, et al. Hypertension in Egypt: a systematic review. *Curr Hypertens Rev* 2014; 10(3): 134–141.
3. Wong T and Mitchell P. The eye in hypertension. *Lancet* 2007; 369(9559): 425–435.
4. Gundogan FC, Isilak Z, Erdurman C, et al. Multifocal electroretinogram in mild to moderate essential hypertension. *Clin Exp Hypertens* 2008; 30(5): 375–384.
5. Browning DJ and Lee C. Relative sensitivity and specificity of 10-2 visual fields, multifocal electroretinography, and spectral domain optical coherence tomography in detecting hydroxychloroquine and chloroquine retinopathy. *Clin Ophthalmol* 2014; 8: 1389–1399.
6. Hood DC, Odel JG, Chen CS, et al. The multifocal electroretinogram. *J Neuroophthalmol* 2003; 23(3): 225–253.
7. Hood DC, Bach M, Brigell M, et al.; International Society For Clinical Electrophysiology of Vision. ISCEV standard for clinical multifocal electroretinography (mfERG) (2011 edition). *Doc Ophthalmol* 2012; 124(1): 1–13.
8. Bach M and Poloschek CM. Electrophysiology and glaucoma: current status and future challenges. *Cell Tissue Res* 213; 353(2): 287–296.
9. Wong TY and McIntosh R. Hypertensive retinopathy signs as risk indicators of cardiovascular morbidity and mortality. *Br Med Bull* 205; 73(1): 57–70.
10. Besharati MR, Rastegar A, Shoja MR, et al. Prevalence of retinopathy in hypertensive patients. *Saudi Med J* 2006; 27(11): 1725–1728.
11. Chatterjee S, Chattopadhya S, Hope-Ross M, et al. Hypertension and the eye: changing perspectives. *J Hum Hypertens* 2002; 16(10): 667–675.
12. Bearse MA and Ozawa GY. Multifocal electroretinography in diabetic retinopathy and diabetic macular edema. *Curr Diab Rep* 2014; 14(9): 526.
13. Faria MY, Sousa DC, Mano S, et al. Multifocal electroretinography in assessment of macular function after internal limiting membrane peeling in macular hole surgery. *J Ophthalmol*. Epub ahead of print March 2019. DOI: 10.1155/2019/1939523.
14. Tzekov RT, Gerth C and Werner JS. Senescence of human multifocal electroretinogram components: a localized approach. *Graefes Arch Clin Exp Ophthalmol* 2004; 242(7): 549–560.
15. Gränse L, Ponjavic V and Andréasson S. Full-field ERG, multifocal ERG and multifocal VEP in patients with retinitis pigmentosa and residual central visual fields. *Acta Ophthalmol Scand* 2004; 82(6): 701–706.
16. Kanski JJ and Bowling B. *Kanski's clinical ophthalmology: a systematic approach*. London: Elsevier Health Sciences, 2015, p. 557.
17. Yu T, Mitchell P, Berry G, et al. Retinopathy in older persons without diabetes and its relationship to hypertension. *Arch Ophthalmol* 1998; 116(1): 83–89.
18. Ibrahim YW, Bots ML, Mulder PG, et al. Number of perifoveal vessels in aging, hypertension, and atherosclerosis: the Rotterdam Study. *Invest Ophthalmol Vis Sci* 1998; 39(6): 1049–1053.
19. Curcio CA, Millican CL, Allen KA, et al. Aging of the human photoreceptor mosaic: evidence for selective vulnerability of rods in central retina. *Invest Ophthalmol Vis Sci* 1993; 34(12): 3278–3296.