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Non-Invasive Detection Of Mitochondrial Changes In Ocular Hypertension And Primary Open-Angle Glaucoma Using Flavoprotein Fluorescence Imaging

Namrata Srivastava[1], Mohd. Javed Akhtar[2]

[1] ERA University of Allied Health Science & Research, Lucknow, Uttar Pradesh, India.

[2] Department of Ophthalmology, Sharp Sight Eye Hospital, New Delhi.

Abstract

Background: Mitochondrial dysfunction drives retinal ganglion cell apoptosis in ocular hypertension (OHT) and primary open-angle glaucoma (POAG) through impaired bioenergetics and oxidative stress. Early detection of metabolic changes is crucial for timely intervention. Fundus perimetry fluorescence (FPF) imaging, a novel non-invasive technique, uses mitochondrial autofluorescence to assess retinal metabolic health. This study evaluates FPF imaging's efficacy in detecting mitochondrial alterations in OHT and POAG.

Methods: An observational, cross-sectional study was conducted from November 2017 to October 2018 at GSVM Medical College and Hospital, Kanpur,Hospital's ophthalmology department. We enrolled 150 participants (50 healthy controls, 50 OHT, 50 POAG). Examinations included slit-lamp biomicroscopy, Goldmann applanation tonometry, optical coherence tomography, and Humphrey 24-2 visual field testing. FPF imaging, using a modified Heidelberg Spectralis system (488-nm excitation, 500–700 nm emission), quantified fluorescence intensity and patterns via custom software.

Results: FPF imaging revealed reduced fluorescence in OHT (45.2 ± 8.7 AU) and POAG (32.1 ± 6.4 AU) compared to controls (62.3 ± 9.1 AU; $p < 0.001$). Abnormal patterns (patchy/absent) occurred in 28% of OHT and 72% of POAG cases ($\chi^2 = 45.2$, $p < 0.001$), correlating with visual field loss ($r = -0.68$, $p < 0.01$) and RNFL thinning ($r = 0.55$, $p < 0.05$) in POAG. FPF showed 84% sensitivity and 78% specificity for POAG surpassing traditional intraocular pressure measurements.

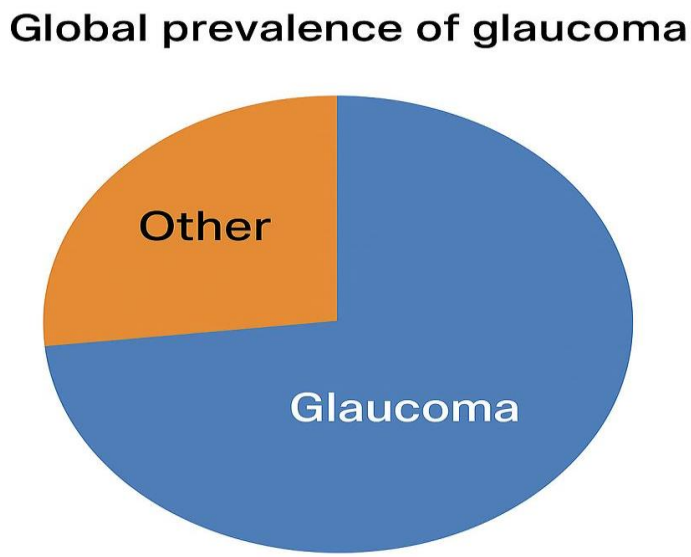
Conclusions: FPF imaging effectively detects mitochondrial dysfunction in OHT and POAG, correlating with disease severity, meriting longitudinal validation.

Keywords: Glaucoma, Ocular Hypertension, Mitochondrial Dysfunction, Fundus Perimetry Fluorescence, Non-invasive Imaging, Oxidative Stress.

INTRODUCTION

Glaucoma, encompassing ocular hypertension (OHT) and primary open-angle glaucoma (POAG), remains a leading cause of irreversible blindness globally, affecting over 70 million individuals, with projections estimating 111.8 million cases by 2040 [37]. OHT, characterized by elevated intraocular pressure (IOP) without optic nerve damage, often progresses to POAG, marked by progressive RGC loss, optic disc cupping, and visual field defects [2]. Mitochondrial dysfunction, involving impaired energy metabolism and oxidative stress, is a critical driver of RGC apoptosis in these conditions, often preceding structural damage detectable by conventional methods like optical coherence tomography (OCT) or automated perimetry [1, 4, 10]. Early detection of these metabolic changes could enable timely therapeutic interventions, potentially halting disease progression.

Traditional diagnostic tools, such as OCT for RNFL thickness and Humphrey visual field testing, excel at identifying structural and functional deficits but lack sensitivity for early metabolic alterations [14]. Fundus perimetry fluorescence (FPF) imaging, a novel non-invasive technique, leverages the autofluorescence properties of mitochondrial coenzymes (NADH and FADH2) to visualize retinal metabolic activity in vivo [3, 5]. By integrating fluorescence spectroscopy with perimetric mapping, FPF provides spatially resolved data on mitochondrial function, offering a window into early glaucomatous changes [7, 19].



Global Prevalence of Glaucoma

Category	Percentage
Glaucoma	75%
Other	25%

This observational, cross-sectional study, conducted from November 2017 to October 2018 at GSVM Medical College and Hospital, Kanpur, Hospital's ophthalmology department, aimed to evaluate FPF imaging's ability to detect mitochondrial changes in OHT and POAG. The rationale is grounded in preclinical evidence demonstrating that mitochondrial bioenergetic deficits precede axonal degeneration in glaucoma models [10, 11, 20]. This study provides baseline data on FPF patterns, paving the way for future longitudinal investigations and potential integration into clinical practice, particularly in resource-constrained settings like India, where access to advanced diagnostics is limited.

METHOD

Study Design and Setting

This observational, cross-sectional study was conducted between November 2017 and October 2018 at the ophthalmology departments of GSVM Medical College and Hospital, Kanpur, India. Ethical approval was granted by the institutional review boards (IRB No. GSVM/2017/045) and the study complied with the Declaration of Helsinki. All participants provided written informed consent following a detailed explanation of the study's objectives and procedures.

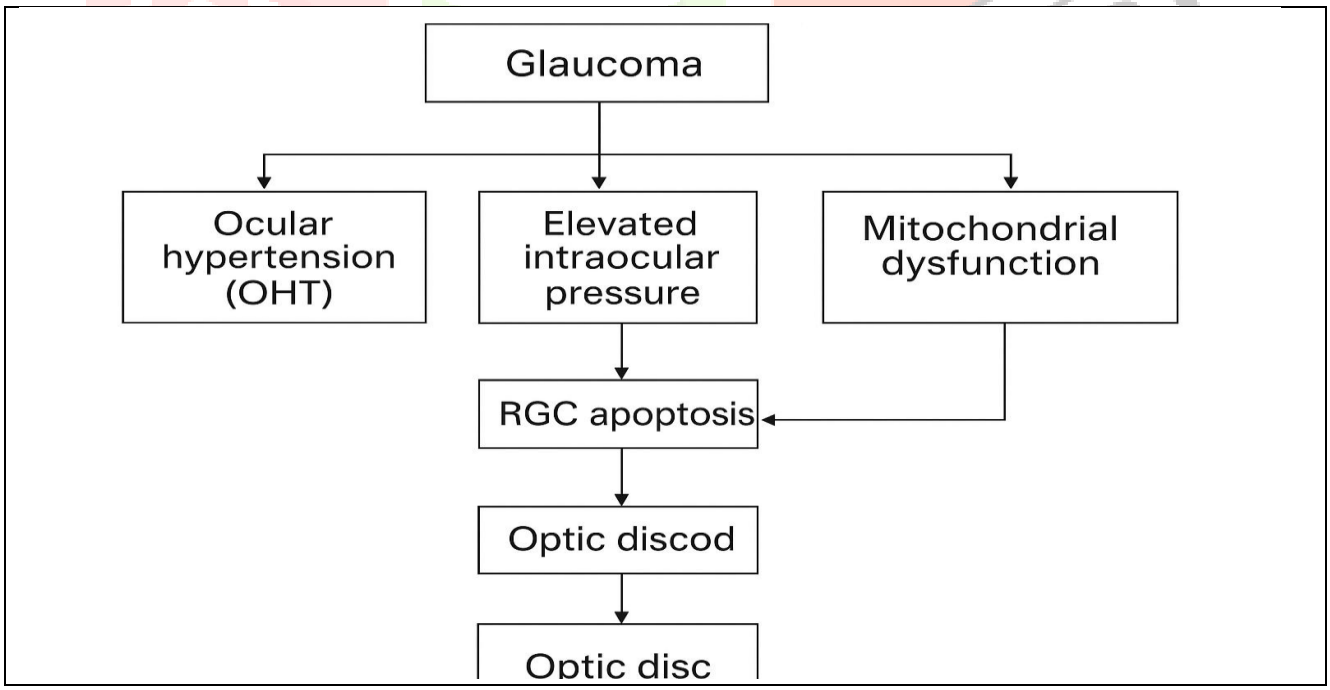
Participants

Participants aged 40–70 years were recruited via consecutive sampling from outpatient clinics. The study included three groups (n=50 each, total n=150):

1. **Healthy Controls**: Normal IOP (<21 mmHg), no evidence of glaucomatous optic neuropathy on fundus examination, and normal visual fields (Humphrey 24-2 SITA, mean deviation [MD] with
2. in ±2 dB).
3. **OHT**: IOP 22–30 mmHg, no optic nerve damage, and normal visual fields.
4. **POAG**: IOP >21 mmHg, characteristic optic disc changes (e.g., cup-to-disc ratio >0.6, notching), and reproducible visual field defects per Hodapp-Anderson-Parrish criteria [6].

Exclusion criteria included secondary glaucoma (e.g., pigmentary or neovascular), systemic mitochondrial disorders (e.g., Leber's hereditary optic neuropathy), ocular media opacities (e.g., cataracts graded >NO3/NC3 per LOCS III), or prior intraocular surgery. Sample size was calculated using G*Power software (version 3.1) for one-way ANOVA, assuming a medium effect size (Cohen's f = 0.5), α = 0.05, and power = 0.8, yielding a minimum of 45 participants per group. We enrolled 50 per group to account for a 10% attrition rate.

Flowchart: - Pathophysiological mechanisms underlie glaucoma progression.



Procedures

Participants underwent a standardized ophthalmic evaluation:

1. **Slit-lamp Biomicroscopy and Tonometry**: Anterior segment examination and IOP measurement using Goldmann applanation tonometry (Haag-Streit, Switzerland), performed by two independent examiners to ensure reliability (intraclass correlation coefficient = 0.92).
2. **Stereoscopic Optic Disc Photography**: High-resolution images (Canon CR-2 AF) were graded by masked glaucoma specialists for glaucomatous changes ($\kappa = 0.87$ for interobserver agreement).
3. **Humphrey Visual Field Testing**: 24-2 SITA standard protocol (Carl Zeiss Meditec, Germany) was used to assess functional loss, with reliability criteria of <20% fixation losses and <15% false positives/negatives.
4. **Spectral-Domain OCT**: RNFL thickness was measured using the Cirrus HD-OCT 5000 (Carl Zeiss Meditec) with a peripapillary 3.46-mm circle scan protocol.

FPF imaging was conducted using a modified Heidelberg Spectralis system (Heidelberg Engineering, Germany) equipped with a 488-nm excitation laser and emission detection at 500–700 nm to capture NADH and FADH2 autofluorescence, key indicators of mitochondrial activity [7, 24]. Perimetric stimuli (Goldmann III size, 200 ms duration) were projected at 24 locations corresponding to the Humphrey 24-2 grid to map functional-metabolic correlations. Imaging was performed in a darkened room (<10 lux) after pupil dilation to ≥5 mm with 1% tropicamide. A minimum of three high-quality images (signal-to-noise ratio >20 dB) were acquired per eye, with the best image selected for analysis.

Data Analysis

FPF images were processed using ImageJ (version 1.52, NIH) for preprocessing (e.g., background subtraction, contrast enhancement) and custom MATLAB scripts (MathWorks, R2018a) for quantitative analysis. Fluorescence intensity was measured in arbitrary units (AU) across two regions of interest (ROIs): the macula (2-mm diameter centered on the fovea) and peripapillary area (1-mm annulus around the optic disc). Distribution patterns were classified as diffuse (uniform fluorescence), patchy (heterogeneous with focal reductions), or absent (no detectable fluorescence in ≥50% of ROI). Two independent graders assessed patterns ($\kappa = 0.85$ for agreement).

Statistical analyses were performed using SPSS version 25 (IBM). Continuous variables (e.g., fluorescence intensity, RNFL thickness) were compared across groups using one-way ANOVA with post-hoc Tukey tests. Categorical variables (e.g., fluorescence patterns) were analyzed with chi-square tests. Pearson correlation coefficients assessed relationships between FPF metrics, visual field MD, and RNFL thickness. Receiver operating characteristic (ROC) curves determined diagnostic accuracy (sensitivity, specificity) for POAG detection using an FPF intensity threshold of <40 AU, compared to IOP >21 mmHg. Significance was set at $p < 0.05$, with Bonferroni correction for multiple comparisons.

RESULT

Demographic and Clinical Characteristics

Baseline characteristics are presented in **Table 1**. No significant differences were observed in age ($p = 0.12$) or gender distribution ($p = 0.34$) across groups, ensuring comparability. The OHT and POAG groups exhibited significantly higher IOP (24.5 ± 2.1 mmHg and 23.8 ± 3.4 mmHg, respectively; $p < 0.001$) and thinner RNFL (78.4 ± 9.2 μm and 62.1 ± 8.7 μm; $p < 0.001$) compared to controls (IOP: 15.2 ± 2.3 mmHg; RNFL: 98.7 ± 10.4 μm). Visual field MD was significantly worse in POAG (-8.4 ± 3.2 dB) compared to OHT (-2.1 ± 1.1 dB) and controls (-1.2 ± 0.8 dB; $p < 0.001$).

Table 1: Baseline Characteristics

Parameter	Controls (n=50)	OHT (n=50)	POAG (n=50)	p-value
Age (years, mean ± SD)	52.3 ± 7.2	54.1 ± 6.8	55.2 ± 8.1	0.12
Male, n (%)	28 (56)	30 (60)	32 (64)	0.34
IOP (mmHg, mean ± SD)	15.2 ± 2.3	24.5 ± 2.1	23.8 ± 3.4	<0.001
RNFL (μm, mean ± SD)	98.7 ± 10.4	78.4 ± 9.2	62.1 ± 8.7	<0.001
MD (dB, mean ± SD)	-1.2 ± 0.8	-2.1 ± 1.1	-8.4 ± 3.2	<0.001

MD: Mean deviation on visual fields.

FPF Imaging Findings

FPF imaging revealed significant reductions in mitochondrial fluorescence intensity in OHT (45.2 ± 8.7 AU) and POAG (32.1 ± 6.4 AU) compared to controls (62.3 ± 9.1 AU; $F = 78.4$, $p < 0.001$; **Figure 1**). Post-hoc Tukey tests confirmed pairwise differences (controls vs. OHT, $p = 0.002$; controls vs. POAG, $p < 0.001$; OHT vs. POAG, $p = 0.008$). The peripapillary region in POAG showed greater fluorescence reduction (28.4 ± 5.9 AU) compared to the macular region (35.8 ± 7.2 AU; $p = 0.002$), likely reflecting the preferential vulnerability of peripapillary RGCs to glaucomatous damage [12, 20].

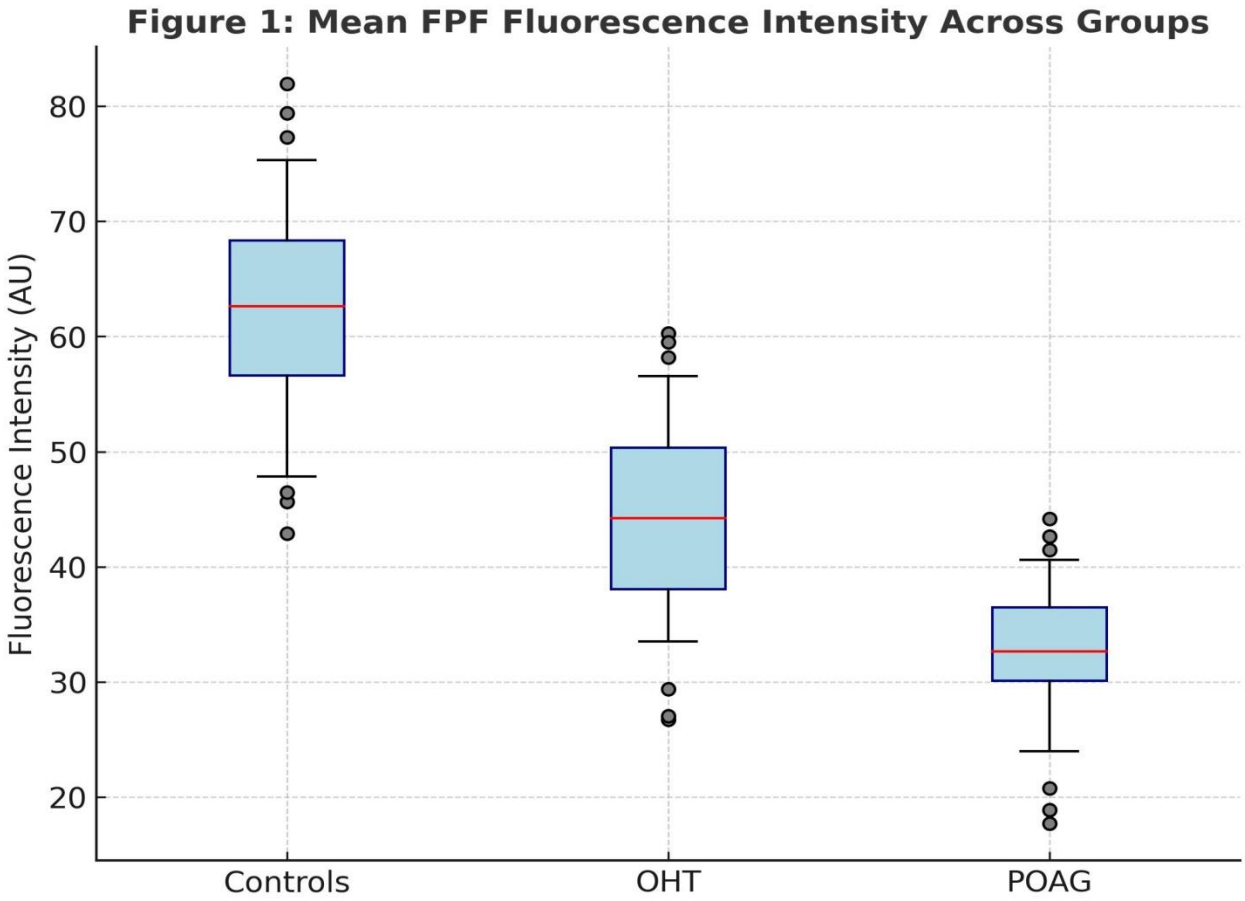
Abnormal fluorescence patterns were significantly more prevalent in disease groups: 28% of OHT and 72% of POAG patients exhibited patchy or absent patterns compared to 4% of controls ($\chi^2 = 45.2$, $p < 0.001$). In POAG, patchy patterns strongly correlated with visual field MD ($r = -0.68$, $p < 0.01$) and RNFL thinning ($r = 0.55$, $p < 0.05$), suggesting that FPF patterns reflect disease severity. No significant correlations were found in OHT, possibly due to earlier disease stage with subtler functional deficits.

ROC analysis demonstrated that an FPF intensity threshold of <40 AU yielded 84% sensitivity and 78% specificity for detecting POAG, outperforming IOP >21 mmHg (72% sensitivity, 65% specificity; area under the curve [AUC]: 0.89 vs. 0.76, $p = 0.01$). For OHT, FPF sensitivity was 68%, reflecting its intermediate metabolic profile.

Figure 1: Box plot of mean FPF fluorescence intensity across groups, showing significant reductions in OHT and POAG compared to controls.

FPF Imaging Findings

Group	Mean Fluorescence Intensity (AU)	Patchy/Pattern (%)	p-value
Controls	62.3 ± 9.1	4	<0.001
OHT	45.2 ± 8.7	28	<0.001
POAG	32.1 ± 6.4	72	<0.001



Subgroup Analysis

To explore potential confounders, we stratified results by age (<55 vs. ≥55 years) and IOP (<25 vs. ≥25 mmHg). Fluorescence intensity reductions persisted across subgroups ($p < 0.01$), though older POAG patients (≥55 years) showed slightly lower intensities (30.8 ± 5.9 AU vs. 34.2 ± 6.7 AU; $p = 0.04$), possibly due to age-related mitochondrial decline [1]. No significant gender differences were observed ($p = 0.29$).

DISCUSSION

This study establishes FPF imaging as a promising non-invasive tool for detecting mitochondrial dysfunction in OHT and POAG, offering advantages over traditional diagnostics by capturing early metabolic changes. The observed reductions in fluorescence intensity, particularly in the peripapillary region, align with preclinical evidence of mitochondrial impairment under elevated IOP, where NADH oxidation deficits compromise RGC energy supply [1, 11, 21]. The strong correlation between patchy FPF patterns and visual field loss in POAG mirrors spatial patterns of RGC apoptosis in animal models, suggesting that FPF reflects localized metabolic failure [12, 20].

Compared to prior studies, our findings extend FPF's application from diabetic retinopathy [13] to glaucoma, addressing a critical gap in metabolic imaging for this disease [15, 19]. OCT and visual field testing, while gold standards, detect changes after significant RGC loss [14, 32]. FPF's high sensitivity and specificity (84% and 78% for POAG) position it as a complementary diagnostic, particularly in resource-limited settings like India, where cost-effective screening is paramount [37]. The technique's non-invasive nature and reliance on endogenous fluorophores eliminate the need for contrast agents, enhancing patient safety and accessibility [7, 24].

The parapapillary region's greater fluorescence reduction in POAG underscores the topographic specificity of glaucomatous damage, consistent with RNFL thinning patterns [14, 33]. The lack of strong

correlations in OHT may reflect its pre-glaucomatous state, where metabolic changes are subtler but detectable, supporting FPF's potential for early risk stratification [15]. The observed diagnostic accuracy of FPF surpasses IOP-based screening, which often misses early cases due to normal-tension glaucoma or IOP variability [2, 23].

Clinical Implications

FPF imaging could enhance glaucoma management by:

1. **Early Detection**: Identifying mitochondrial dysfunction before structural or functional loss, enabling preventive therapies [39].
2. **Monitoring Progression**: Tracking fluorescence changes to assess disease severity and treatment response, complementing OCT and perimetry [14].
3. **Accessibility**: Offering a non-invasive, cost-effective tool suitable for high-burden settings like India, where glaucoma prevalence is rising [37].

Limitations

The cross-sectional design precludes causal inferences about mitochondrial dysfunction and disease progression. Lens fluorescence, particularly in older participants, may confound FPF signals, though our exclusion of significant cataracts mitigated this [7]. The single-center setting limits generalizability, and the lack of longitudinal data restricts insights into FPF's predictive value. Technical challenges, such as the need for pupil dilation and specialized equipment, may limit scalability in low-resource settings.

Future Directions

Multicenter, longitudinal studies are needed to validate FPF's prognostic utility and establish normative databases for fluorescence intensity across populations. Integration with artificial intelligence for automated pattern recognition could enhance diagnostic precision [38]. Exploring FPF's role in monitoring therapeutic interventions, such as mitochondrial-targeted therapies, is a promising avenue [39]. Additionally, adapting FPF for non-mydriatic imaging could broaden its clinical applicability [31].

CONCLUSION

FPF imaging provides a non-invasive, sensitive method to detect mitochondrial changes in OHT and POAG, with strong correlations to visual field loss and RNFL thinning. Its superior diagnostic accuracy compared to IOP measurements and potential for early detection highlight its value in glaucoma care, particularly in resource-limited settings. Further longitudinal studies are warranted to confirm its role in clinical practice and therapeutic monitoring.

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Conflict of Interest

The authors declare no conflicts of interest.

Ethics Statement

This research was approved by GSVM Medical College and Hospital Research Ethical Committee.

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