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Quantifying mitochondrial stress in LHON patients – an exploratory study of flavoprotein autofluorescence

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Abstract

Purpose

Leber Hereditary Optic Neuropathy (LHON) is a primary mitochondrial disorder, where retinal ganglion cell dysfunction typically leads to sequential blindness. Although targeted gene therapies have shown promise, clinical trials have struggled to show efficacy using traditional biomarkers. Flavoprotein autofluorescence (FPF) is able to quantify mitochondrial oxidative stress *in vivo*, and warrants exploration as a potential biomarker for future clinical trials.

Methods

This was a cross-sectional exploratory study examining FPF in 13 LHON patients (24 eyes) and 18 controls (36 eyes). Raw FPF was quantified at the optic nerve head rim (Garway-Heath sectors) and the macula (ETDRS sectors). Corrected values were also calculated accounting for tissue loss. Mixed effects models were used to compare LHON and control groups and to examine other covariables. Correlation with other established metrics was also examined.

Results

Both raw ($t = 5.68$, $p < 0.001$) and corrected ($t = 7.63$, $p < 0.001$) FPF values were significantly higher in LHON patients at the optic nerve head. This was confirmed for all individual rim segments. In contrast, only when corrected for tissue loss was macula FPF significantly higher ($t = 6.65$, $p < 0.001$). Symptom duration, disease severity and age all variably influenced regression models. FPF was variably correlated with other visual metrics.

Conclusions

FPF is significantly higher in LHON patients than controls both at the optic nerve head rim and central macula. Tissue loss is a critical factor which must be taken into account. FPF may offer additional information beyond traditional biomarkers, and shows good structure-function correlation.

Introduction

Leber Hereditary Optic Neuropathy (LHON, OMIM: 535000) is a mitochondrial disorder typically leading to severe sequential visual loss in young patients. [1] It affects 1 in 30,000–50,000 people in European cohorts, and approximately 1 in 9000 within these populations are carriers [2, 3]. As 95% of cases arise from three discrete mutations in mitochondrial DNA (mtDNA: m.3460A > G, m.11778G > A, m.14484T > C) [4], targeted genetic therapies have been developed. [1]

Clinical trials in LHON have largely used recovery of best corrected visual acuity [1], supported by a large natural history study examining candidate biomarkers in LHON [5]. However, interpreting these trials has not been straightforward. The variable natural history, sequential nature of visual loss and potential for spontaneous visual improvement [1, 6], coupled with unanticipated visual improvement in contralateral untreated eyes has called for a reevaluation of current study design and for more objective trial endpoints. [1]

Flavoprotein fluorescence (FPF) is an emerging metabolic biomarker which is yet to be evaluated in LHON patients. When mitochondria undergo oxidative stress, flavoproteins become oxidized. [7] Oxidized flavoproteins emit a green fluorescence (~ 530nm) when excited by blue light (~ 460nm), a phenomenon termed FPF. As flavoproteins are intimately linked to electron transport chain enzymes, FPF affords real-time assessment of oxidative stress and is a surrogate for mitochondrial dysfunction. Further, FPF intensity rises before apoptosis occurs, is quantifiable, is distinct from other retinal fluorophores and is attenuated by N-acetyl-cysteine suggesting sensitivity to treatment response. [7–9] It is therefore an attractive trial endpoint as it is non-invasive, quantifiable, objective, easily interpretable, and most notably it directly measures the site of cellular dysfunction and therapeutic targets with high conceptual validity. [10] FPF has been shown to be abnormal in primary open angle glaucoma [11, 12] and ocular hypertension [11] and most recently optic disc drusen [13]. Herein we explore FPF as a possible biomarker in LHON and discuss its potential role as a clinical trial endpoint.

Methods

Ethics approval

was granted by local HREC, and the study was carried out according to the Declaration of Helsinki. LHON patients were identified from a pre-existing database across two tertiary clinics in Sydney, Australia. All patients had genetically confirmed LHON with likely pathogenic or pathogenic mutations according to ACMG guidelines [14], and included eyes required evidence of optic neuropathy without confounding ocular comorbidities. Control patients were also recruited, and all had a normal ocular examination. Informed consent was gained from all participants.

Data collection

All patients underwent visual acuity (best corrected visual acuity (BCVA), spectral domain optical coherence tomography (SD-OCT) and FPF imaging. Subjective refraction was performed prior to testing best corrected high contrast visual acuity. A logarithm of the minimum angle of resolution (LogMAR) chart was used. Where a patient had vision less than 1.0 LogMAR, they were brought to a 1m distance. If this failed, LogMAR values were derived from “count fingers”, “hand movements” and “light perception” as per Lang et al. [15] Patients were dilated prior to SD-OCT testing. Images were captured using the Heidelberg Spectralis (Heidelberg Engineering, Heidelberg, Germany). Peripapillary retinal nerve fibre layer (RNFL) and macular were imaged, and were subdivided according to six-region Garway-Heath

maps and posterior pole ETDRS sectors including layer segmentation respectively. All scans were assessed for quality and clarity, with segmentation adjusted as required.

FPF image acquisition using the OcuMet Beacon (OcuSciences Inc. Ann Arbor, MI) has been described in detail previously [8]. Standardized testing procedures included machine calibration, mesopic lighting conditions, patient dilation and correct patient alignment. A 60^{o} x 21.5^{o} infrared image was taken to assess quality and illumination, and to facilitate registration of the ONH rim and macula. A brief flash of blue (455-470nm) light was followed by a 17^{o} x 21.5^{0} FPF image generated using a narrow band pass filter (530-540nm) to detect the emitted green autofluorescence. Images were taken at the central macula or optic nerve head (ONH) and were repeated once in each location. Images with poor quality indices (FPF quality score < 50, shadow artefact, or poor centration or fixation) were removed from the analysis.

Statistical Analysis

Image analysis by OcuSciences Inc. yielded “raw” FPF values for both the ONH rim and the macula which included adjustment for lens FPF (see Fig. 1). Global and sectoral results were available for each. The ONH rim was automatically identified and referenced from the matching infrared image, and subdivided into six-location Garway-Heath sectors. Macular FPF signal within a 5.5mm diameter circle centered on the fovea was analyzed. Only the inner five ETDRS sectors were used, as outer sectors typically lay partly outside the imaged area. We then calculated “corrected” values to adjust for the degree of tissue loss by dividing the raw score by OCT structural data. Global RNFL and sectoral RNFL OCT thicknesses were used to adjust the corresponding optic disc FPF values. Similarly, global macular ETDRS GCL volume, and ETDRS quadrant GCL thickness were used to correct the corresponding macula FPF values.

Statistical analysis used R 4.5.1 (R core team, 2025), and all data was screened for completeness and outliers. Both eyes of each subject were included for analysis where possible. Baseline and demographic data were compared between groups using two-sided Fisher’s exact test for categorical data, and Welch t-test for continuous data. A linear mixed effects model was used to compare FPF metrics between LHON patients and controls, while controlling within-subject clustering. Model assumptions were assessed, while age, gender and idebenone use were included as covariables. Findings were reported as estimated effect size with 95% confidence intervals, along with t statistic and p value. Subgroup analysis was then performed on LHON patients to assess correlations between FPF metrics and other variables using Spearman correlation. Mixed effects models were again used to test the effect of idebenone and duration of symptoms on FPF values, correcting for patient age and severity of visual loss. Significance level was set at $p \leq 0.05$ throughout.

Results

A total of 13 LHON patients (24 eyes) and 18 controls (36 eyes) were recruited. Baseline demographics and comparison statistics are detailed in Table 1. Almost half of LHON patients (6/13) were using

idebenone, a rate significantly higher than controls. There were no other significant differences in baseline characteristics between groups.

Table 1
– Baseline characteristics of recruited LHON patients and controls.

Total patients (n)	**LHON**	**Controls**	**Comparison p value**
Total eyes (N)	**13**	**18**	
	24[a]	**36**	
Age	40.5 ± 15.5	40.9 ± 14.9	P = 0.94
Gender (female/total)	5/13	12/18	P = 0.13
Ethnicity (n)	10	12	P = 0.56[c]
- White	1	6	
- Asian	2	0	
- Other[b]			
Comorbidities (/n)	1/13	0/18	P = 0.42
- DM	1/13	1/18	P = 0.84
- Smoking	3/13	0/18	P = 0.06
- HTN			
Idebenone use	6/13	0/18	P = 0.005
Spherical Equivalent	-0.17 ± 1.21	-0.62 ± 1.68	P = 0.39

Age and spherical equivalent reported as mean +/- standard deviation. Categorical variables represented as counts. DM – diabetes mellitus, HTN – hypertension. [a] Two LHON patients had one eye excluded due to amblyopia (N = 1) or visually significant cataract (N = 1). [b] One patient was of Aboriginal background, and another Middle-Eastern. [c] Calculated as European vs non-European. There was also no significant difference between rates of individual Ethnic groups.

Mixed effect regression models were used to compare LHON patients and controls at the optic disc (see Fig. 2 and supplementary table 1) and macula (Fig. 3 and supplementary table 2). At the disc, both raw and corrected FPF values were significantly higher in LHON patients than controls ($p < 0.001$) in all locations (global and sectors). Corrected FPF values tended towards higher t values than did raw FPF values in most locations, but this was not the case in superonasal and nasal sectors. By way of reference, comparing BCVA between groups in the same way yielded a similar level of significant difference ($t = 6.31$, $p < 0.001$), while global macular GCL volume showed a greater level of difference ($t = 13.61$, $p < 0.01$) between groups.

At the macula, we found no significant difference in raw FPF values between LHON patients and controls in any location. When corrected for OCT GCL loss, we found significantly higher ($p < 0.001$) FPF readings in LHON patients compared to controls in all locations. T values for corrected macula FPF analysis were comparable to those of the ONH.

Univariate correlation between FPF and other variables was restricted to LHON patients (Table 2). These were performed at the subject (not individual eye) level, with the mean value taken for paired variables where applicable. We found weak correlation between FPF values and BCVA. This correlation was positive for optic disc FPF values, and negative for macular FPF values. Strong to very strong negative correlations were found between OCT (macular GCL and RNFL) and disc FPF (raw and corrected) metrics. This suggests FPF signal at the disc increases with OCT thinning. Log-duration of symptoms showed strong positive correlation with disc FPF metrics, and variable positive correlation with macular FPF metrics. In general, OCT metrics and symptom duration were better correlated with disc FPF values than macula FPF values. Importantly for subsequent analysis, age and log duration of symptoms were not correlated. It should be noted that corrected FPF values incorporate OCT data within them, so should be interpreted cautiously.

Table 2
– Spearman correlation coefficients between different variables.

Age	**Mean FPF disc**	**Mean FPF disc corrected**	**Mean FPF mac**	**Mean FPF mac corrected**
	0.26	**0.14**	**0.28**	**0.49**
Mean BCVA	0.21	0.36	-0.32	-0.08
Log-duration of symptoms	0.66	0.82	0.25	0.61
Mean mac GCL volume	-0.88	-0.79	0.06	-0.51
Mean disc RNFL	-0.72	-0.89	0.14	-0.33

Shaded cells identify scenarios where the corrected FPF composite metric contains the variable it is being correlated with.

Mixed-effects models were again used to assess the impact of idebenone use, age, symptom duration and BCVA (as a surrogate for disease severity) on FPF values (see Supplementary Table 3). We were unable to detect a significant effect of idebenone use on FPF values in our LHON cohort. BCVA was significantly positively correlated with raw disc FPF ($t = 2.13$, $p = 0.047$), but not for other FPF metrics. There was stronger correlation between BCVA and disc FPF than macula FPF. Log-duration of symptoms was also positively correlated with raw disc FPF ($t = 2.43$, $p = 0.036$), and trended towards a significant positive correlation for corrected FPF disc ($t = 1.83$, $p = 0.10$) and corrected FPF macula ($t = 1.96$, $p = 0.11$). These effects were independent of patient age.

Discussion

To our knowledge, this is the first report to assess FPF in a LHON population. We have found a clear difference between LHON patients and controls at the ONH, as well as at the macula when tissue loss is controlled for. ONH FPF was significantly greater at all sectors and globally for both raw and corrected values. At the macula, there was no significant difference in raw values between LHON patients and controls. However, when tissue loss was accounted for, this revealed a significantly higher corrected FPF in LHON patients at all inner ETDRS locations and globally. We found strong univariate correlation between disc FPF values and structural OCT metrics at the ONH and macula at an individual level. When individual eyes were analyzed with regression models, a positive correlation between raw ONH FPF and BCVA was seen. Raw ONH was also found to be positively correlated with log-symptom duration both with univariate and multivariate models. Correlations between macular FPF metrics were much weaker.

There is a growing need to assess novel biomarkers for future LHON trials. The only long term prospective longitudinal study available [5] found 18% of LHON patients experienced spontaneous visual recovery of > 15 ETDRS letters up to 6 years after visual loss. BCVA was thus considered the most suitable trial end-point due to its sensitivity to detect change in visual function and therefore potential treatment response. This rate of spontaneous recovery was supported by the pooled findings of Newman et. al. [16] in 2020, along with the established sequence of visual nadir, followed by a dynamic phase and finally stability of BCVA. Natural history data for other non-BCVA biomarkers is variable. Yang et. al. [17] found changes in BCVA in LHON patients to correlate with 30 – 2 visual field mean deviation and VFI as well as P100 metrics. Several variables have been shown to decline prior to BCVA loss, including SS-PERG [18, 19] and OCT GCIPL [19]. Others have shown that VF [16, 20] and OCT [21, 22] metrics may evolve beyond the classic nadir between 4–6 months.

Translating this background to the clinical trial setting, the potential issues with BCVA become more apparent. In 2011, the RHODOS study [23] investigated the effect of idebenone in LHON patients using best recovery of visual acuity as its primary end point. Despite enrolling 85 LHON patients and anecdotal reports of treatment efficacy, the intention to treat analysis failed to reach statistical significance. Post-hoc analysis found significant benefit in for those with discordant visual acuities and provides key evidence for the use of idebenone in LHON patients. However it also highlights the difficulties and potential pitfalls of BCVA in this population. More recently, the LEROS study [24] found a greater proportion of idebenone-treated LHON patients achieving a clinically relevant benefit from 12 months of idebenone treatment. What defines “clinically relevant recovery” or “clinically relevant benefit” is of course debatable, and is one of the shortcomings of an analysis based on proportions. Similarly, intravitreal allotopic intravitreal gene therapy trials have had their own challenges. In 2024, Lam [1] cited spontaneous visual improvement, the sequential nature of visual loss and the subjectivity of BCVA as key challenges encountered thus far. Three of the largest trials, RESCUE [25], REVERSE [26] and REFLECT [27] failed to meet their primary end point of BCVA improvement following intravitreal injection. Contralateral control eyes [25, 26] and those treated with placebo [27] also showed BCVA improvement, which has also been found in unrelated studies [28]. The reliance on BCVA extends to other

interventional studies for alternative therapies such as brimonidine [29], stem cell therapy [30], cyclosporine [31] and elamipretide [32]. Lam [1] suggests that future trials would benefit from untreated control LHON patients, however this would also affect recruitment and ethical considerations. As the superiority of BCVA is not clear cut, alternative biomarkers such as FPF may also offer solutions, which highlights the importance of this exploratory study.

In our cohort, raw ONH FPF was significantly higher ($p < 0.001$) across global and all Garway Heath rim sectors, with comparable t statistics across all locations. Correcting for tissue loss inflated these values further, again reaching strong significance in all locations ($p < 0.001$). Despite slightly lower levels of significance compared to corrected metrics, multivariable regression models found raw ONH FPF was positively correlated with BCVA ($p = 0.047$) while corrected values were not. There was also strong univariate correlation between raw ONH FPF and structural metrics of RNFL ($r = -0.72$) and GCL volume ($r = -0.88$). As the corrected ONH FPF did not offer any clear benefit and introduces an additional variable, the global raw ONH FPF is likely the most simple and effective marker of optic nerve FPF. Taken together, raw ONH FPF is significantly higher in LHON patients than controls, and correlates with other markers of visual system structure and function. Although reports of FPF in other optic neuropathies are limited, Zhou et. al. [12] found similar results in POAG eyes when measuring FPF at the ONH rim. POAG eyes had higher FPF readings compared to controls, and were correlated with VF MD, VF PSD and OCT RNFL.

Macular FPF was more complicated than our ONH results. In LHON patients, raw macular FPF was lower at all locations except the fovea, and no location reached statistical significance. However when corrected for tissue loss, all locations showed significantly higher FPF metrics ($p < 0.001$). The importance of correcting for tissue loss has been noted by Geyman et. al. [11], who proposed that tissue loss means fewer mitochondria are able to fluoresce. By correcting for tissue loss (dividing raw FPF by a corresponding structural metric), the resulting “FPF per unit of tissue” offers an alternative metric to raw FPF. Correlation with BCVA was very weak (-0.07), and correlation with structural metrics is inappropriate as the composite value comprises structural OCT results already. Thus, corrected macular FPF was significantly higher in LHON patients than controls, but its utility appears limited given minimal correlation with other markers of visual function as well as the inherent shortcomings of a composite metric.

As a potential clinical trial end-point in LHON studies, several points should be noted. The first is the promising results of raw ONH FPF values in this cohort. The strongly significant results, coupled with strong correlation to existing visual biomarkers supports its relevance. Unlike BCVA it is objective and quantifiable, and unlike structural metrics it affords real-time functional and metabolic information about the target tissue. Strong correlations between particularly ONH FPF and structural OCT metrics supports a structure-function relationship. More than this, it has high conceptual validity as it images the target tissue directly, and measures mitochondrial function which is both the pathologic organelle as well as the direct therapeutic target [10]. The only caveat to its utility may be a relatively higher test-retest variability, with variable registration and vessel masking along with unstable fixation contributing.

Although only performed in one subject, coefficient of variation (CoV) of ONH rim FPF was as 8.2% in a POAG patient compared with 10.5% in a control [12]. Macular FPF CoV was lower – 1.68% in a diseased eye (not defined) and 2.82% in a control eye in a different study [8]. This remains to be assessed in a LHON cohort.

The second is the sensitivity to tissue loss, particularly at the macular. While we did not find any reason to favour macular FPF over ONH FPF, this must be kept in mind and adjusted for if macular values are to be used. It is unclear which marker of tissue loss is best, and the introduction of a second variable carries with it its own issues for a methodological and statistical viewpoint.

The third is a suggestion of how FPF behaves over time. There was a strong correlation between raw ONH FPF and log-duration of symptoms on univariate analysis, and a mixed effects model again found a positive correlation ($t = 2.43$, $p = 0.036$). This suggests that there may be an initial steep increase in FPF which plateaus over time. Although this does not offer much information about initial changes around the time of visual loss, it suggests that FPF values stay elevated long after symptom onset. In other words, there is no descending limb, and suggests spontaneous improvement may be less likely to be seen with ONH FPF than with BCVA. It also suggests that in the acute phase there may be more rapid FPF changes, and that this dynamic phase may be prime for detecting treatment response and optimizing sensitivity.

Conclusion

In a condition where previous trials have rested heavily on BCVA to measure treatment response, FPF offers an objective alternative. Where the vast majority of LHON patients possess BCVA < 6/60 or “off the chart”, the traditional measure of 3 lines of improvement may be less relevant. Instead, a metabolic approach where a therapy can be proven to alter cellular functioning may prove more useful. This study is limited by its small cohort, and the cross-sectional nature limits predictions regarding change over time. Establishing test-retest variability, along with dedicated longitudinal studies will be required to evaluate its utility as a clinical trial endpoint.

Declarations

Competing Interests

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Author Contribution

CO, JG and CF were all responsible for study design and set upCO performed testing, statistical analysis and manuscript preparationCO, JG and CF were all involved in manuscript revisions and final submission

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Data Availability

All data supporting the findings of this study are available within the paper and its Supplementary Information. Results of regression models and covariate effect estimates are available from supplementary tables 1-3.

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Figures

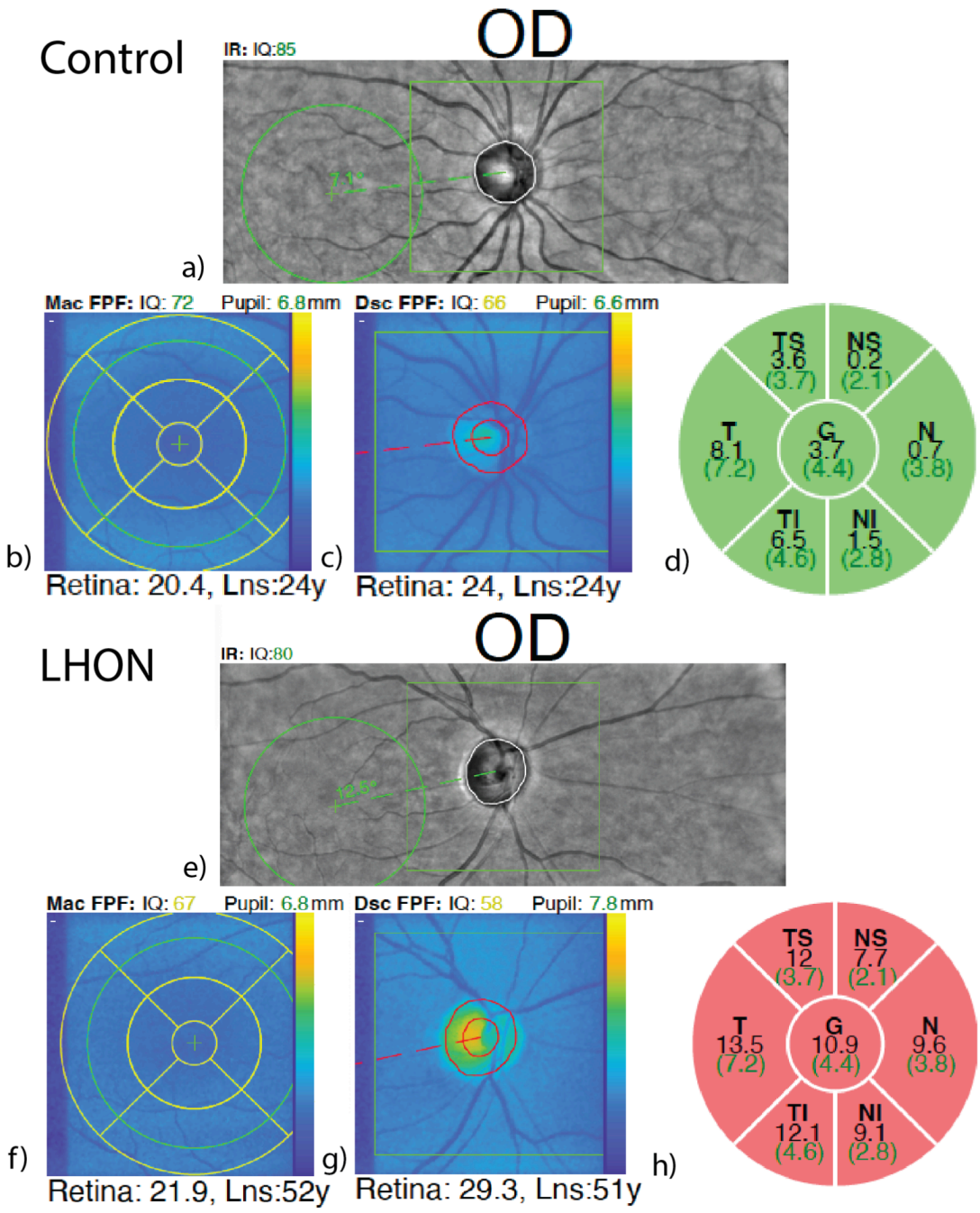


Figure 1

Sample FPF data for the right eye (OD) of a control (top) and LHON (bottom) patient. a) and e) Infrared image showing area imaged for FPF at the ONH (green square) and macula (green circle). b) and f) macula FPF findings, with overlaid ETDRS (yellow) grid. c) and g) ONH rim FPF images, with readings taken from between the inner and outer red concentric n-gons. This was further quantified to give d) and h), with global and 6-location Garway-Heath maps showing the patient's raw FPF intensity (dB gsu) along

with age-matched population average in brackets below. All raw images (a-c, e-g) show image quality (IQ) score. All FPF images (b,c,f,g) depict FPF intensity according to the heat map on right vertical axis, as well as global FPF from within the imaged area ("Retina:") and an estimation of patient age based on lens plane signal ("Lns:")

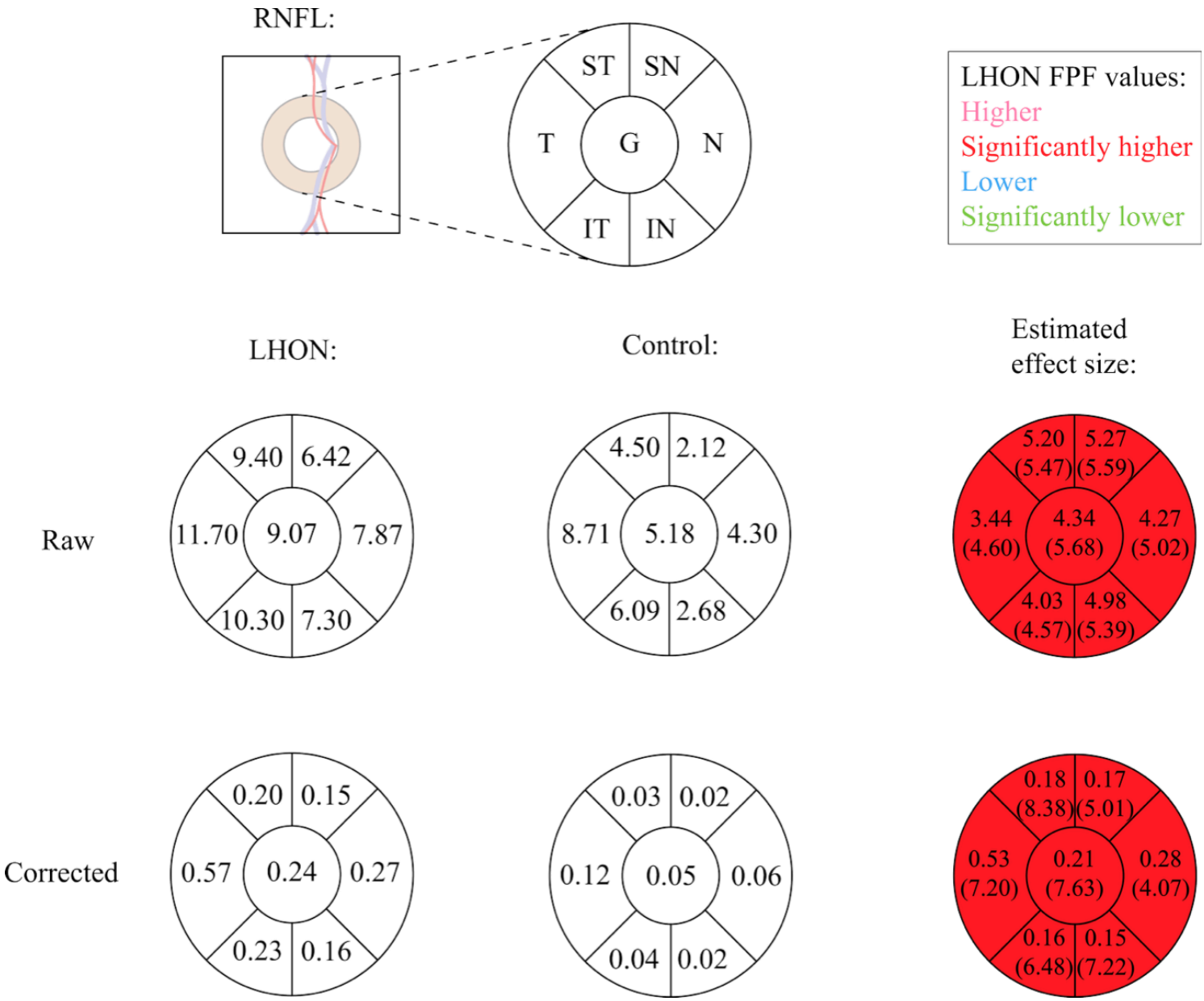


Figure 2

Pictorial representation of FPF values at the optic nerve head. Top schematic shows the Garway-Heath sectors for a right eye, G – global, T – temporal, ST – superotemporal, IT – inferotemporal, N – nasal, SN – superonasal, IN – inferonasal. Below are the FPF values in each sector for raw and corrected values in LHON patients and controls. Right column shows the estimated effect sizes derived from linear mixed effects models, given as effect size (t statistic). A positive value represents a higher FPF value in LHON patients than controls. A larger t statistic represents a greater statistical difference between the two

groups. Sector colours represent the direction and statistical significance of the effect size, as per the key (top right).

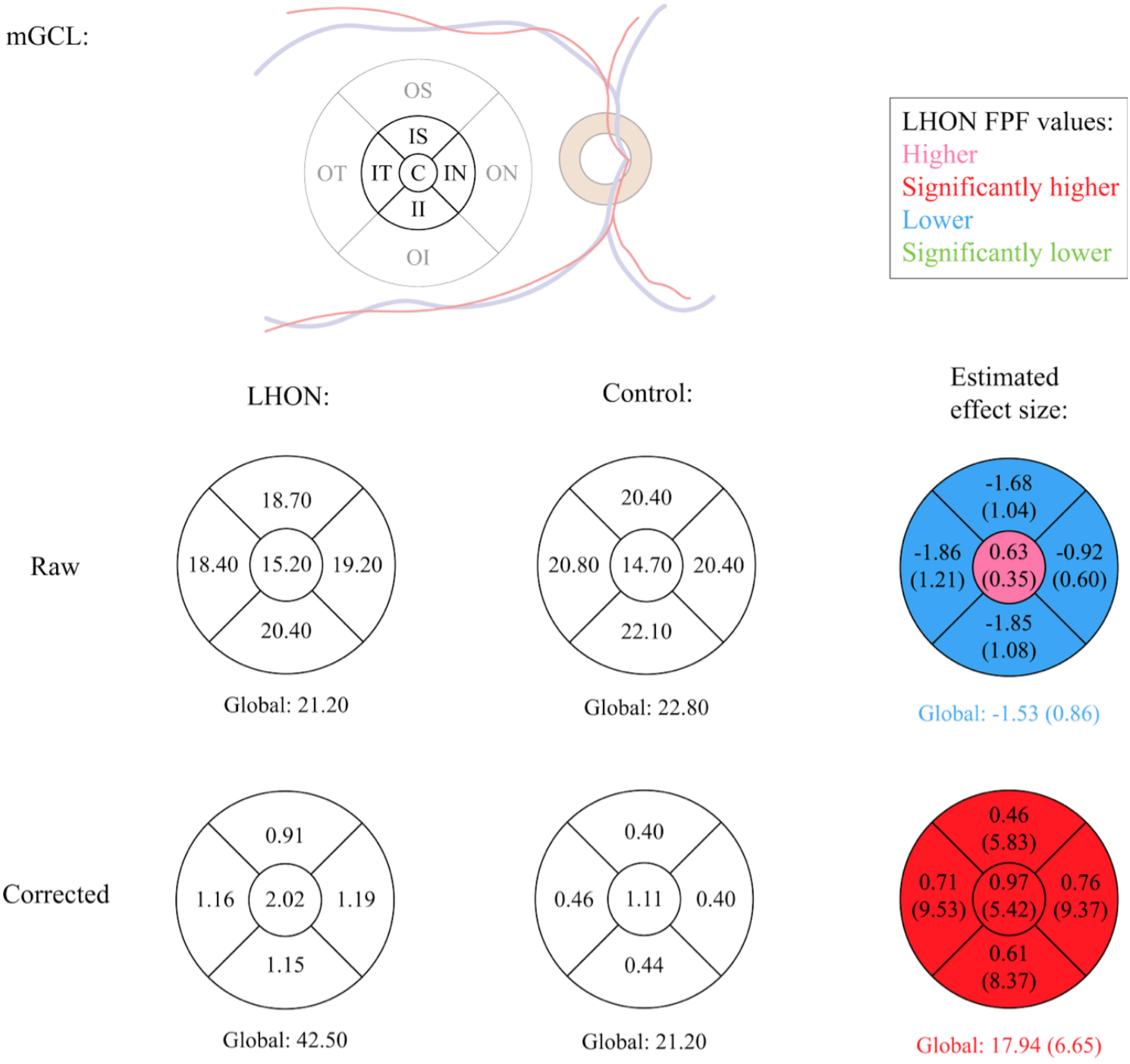


Figure 3

Pictorial representation of FPF values within the macula ganglion cell layer. Top schematic shows the ETDRS grid over the macula of a right eye. C – central, IN – inner nasal, ON – outer nasal, IS – inner superior, OS – outer superior, II – inner inferior, OI – outer inferior, IT – inner temporal, OT – outer temporal. G – global, T – temporal, ST – superotemporal, IT – inferotemporal, N – nasal, SN – superonasal, IN – inferonasal. Below are the FPF values for central and inner ETDRS sectors for raw and corrected values in LHON patients and controls. Right column shows the estimated effect sizes derived

from linear mixed effects models, given as effect size (t statistic). A positive value represents a higher FPF value in LHON patients than controls. A larger t statistic represents a greater statistical difference between the two groups. Sector colours represent the direction and statistical significance of the effect size, as per the key (top right)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- LHONFPFDocumentaTable1.docx
- LHONFPFDocumentaTable2.docx
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