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Effects of supplementation of macular pigment carotenoids on ocular health: a Raman spectroscopic study of human blood serum of glaucoma patients

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Carotenoids are known for their antioxidant and vision protection roles, with dietary supplements often promoted for eye health. An initial trial, the European Nutrition in Glaucoma Management (ENIGMA), assessed macular pigment optical density (MPOD) and other ocular parameters before and after supplementing glaucoma patients with macular pigment (MP) carotenoids. The trial confirmed significant improvements in clinical ocular health. Blood, containing all major dietary carotenoids, serves as an efficient medium for *in vivo* analysis of carotenoids. Raman spectroscopy, an effective analytical tool, was used to measure the impact of supplementation on serum carotenoid levels and their correlation with MPOD and other ocular responses. Serum samples from baseline and 18-month supplemented participants were analysed. An inverse relationship was found between the percentage change in Raman intensity over the supplementation period and baseline Raman serum measurements, indicating greater relative benefits for people with low MPOD/serum carotenoids pre-supplementation. Partial least squares regression (PLSR) was employed to analyse the spectra after pre-processing, and the loadings reflected the carotenoid content and structural profile. MPOD results correlated at all eccentricities, with a coefficient of determination (R^2) of 0.62–0.92 and %Root mean squared error of <44%. Structural, functional, and perceptual parameters also showed good correlation with serum Raman measurements. The results support the ENIGMA trial conclusions, and suggest strategies for optimizing patient responses to supplementation based on baseline carotenoid levels. Additionally, Raman spectroscopy of serum carotenoids shows significant potential as a simple and reliable method for investigating macular pigment carotenoids and assessing patient health.

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1 Introduction

A group of highly pigmented lipophilic compounds known as Carotenoids can be found throughout nature.[1,2] These compounds typically accumulate in carotenoid-rich foods and, when consumed, are absorbed, circulated in blood and deposited in different tissues,[3] from where they perform antioxidant and photoprotective roles in the body.[4] Carotenoids have been used as a measure of nutritional status[5,6] and as an important marker implicated in several diseases, including cancers,[7–10] cardiovascular diseases[11,12] and diabetes.[13–16]

The macular pigment (MP) of the eye is comprised of the carotenoids lutein, zeaxanthin and *meso*-zeaxanthin, and together they form the highest concentration of carotenoids in the body.[17] These constituent carotenoids have been studied extensively for their role in eye health, especially in age-related macular degeneration (AMD)[18] and, more recently, in diabetes[19] cataract[20] and glaucoma.[21] It is therefore important to establish accurate, effective and accessible methods for measuring MP carotenoids, as currently there is no universal standard for measuring MP status in clinical practice and screening for the risk of ocular or neurodegenerative disease related to MP level is not routinely performed.[22,23]

The macular pigment optical density (MPOD), which represents an estimation of the density of macular pigment (MP) in the center of the retina,[24] has been used as an efficient measure of the macular carotenoids. It is obtained by measur-

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ing blue light attenuation by the macular pigment[25] using gold standard methods based on autofluorescence, heterochromatic flicker photometry (HFP) or reflectance.[24,26] These methods have proven effective, but their psychophysical demands can pose considerable limitations, especially when carrying out investigations in the elderly.[27] They are also expensive and not widely accessible.[27,28]

Raman spectroscopy presents as a potential alternative for effectively evaluating the macular pigment. A protocol for using Raman spectroscopy to measure MP carotenoid directly in the living human eye has already been established.[29,30] Even though the method is rapid, repeatable, highly sensitive and specific, and provides an absolute measurement of the macular carotenoids,[30,31] it still requires concentration from the patient (patients have to fixate on a target)[27] which can be a limiting factor especially in the elderly. Additionally, while the method progressed to clinical trials in the US in 2003, the results were not published, and Raman spectroscopy of the eye is still not yet established as a routine technique.[32] Raman spectroscopy of blood/serum on the other hand can provide an efficient alternative approach to routinely monitoring the MP carotenoids through a simple blood test.

Blood contains the MP carotenoids as well as all other major dietary carotenoids, albeit in small quantities.[33,34] These carotenoids can dominate the Raman spectrum of blood serum, however, especially when using visible lasers as source, at whose wavelengths the spectral response of the carotenoids is resonantly or near resonantly enhanced.[35,36]

Initial protocols for monitoring the carotenoid content and structural profile have been established, first in bovine serum albumin (BSA)[37] and then directly in human serum samples.[38] In the latter, participant samples from the ENIGMA (see Section 2.1) clinical trial were used. These were participants with open angle glaucoma (OAG) taking part in the trial, carried out at the Centre for Eye Research Ireland (CERI),[39] who had received supplementation with MP carotenoids for a period of eighteen months. The supplementation with MP carotenoids has been demonstrated to successfully augment the MP in AMD[18,40] and recently in glaucomatous eyes, as demonstrated by the ENIGMA study.[39] The supplementation in the ENIGMA study resulted in a 60% mean increase in macular pigment optical density (MPOD) over the 18-month period. Furthermore, an evaluation of the percentage change indicated that participants with lower baseline MPOD benefitted the most from the increase in MPOD over the supplementation period.[39] Following these findings, Raman spectroscopic analysis of the pre-supplementation baseline and 18-month supplemented blood serum carotenoids from participants of the ENIGMA study was carried out to explore the potential value of using Raman spectroscopy as a reliable alternative for measuring MP carotenoid intake as an indicator of MPOD status in the eye. Although carotenoid concentrations can vary widely in serum due to diet and lifestyle,[41] the results from this initial Raman analysis confirmed a consistent increase in serum carotenoid concentration in supplemented patients, and notably indicated that the structural profile of the serum carotenoids was influenced by the supplementation programme, consistent with increased serum content of the MP carotenoids, lutein, zeaxanthin and *meso*-zeaxanthin.[38] The current study aims to support the initial Raman spectroscopic findings,[38] by further examining the relationship between baseline Raman intensity of serum carotenoids and percentage change over 18 months. Furthermore, partial least squares regression (PLSR) analysis will be employed to examine the correlation between the serum Raman measurements with the MPOD (measured using autofluorescence[24]) results from the ENIGMA trial, to explore the correlation between MP status and carotenoid supplementation, based on Raman spectroscopy of serum.

The ENIGMA study also involved additional exploratory analyses of important ocular functional, structural and perceptual responses to the carotenoid supplementation which reportedly showed no clinically significant changes as a result of the supplementation.[39] These parameters analysed include series of examinations usually carried out in the Optometry clinic to monitor the visual performance of patients and are useful in the diagnosis of glaucoma and other ocular diseases.[42–44]

The functional indicators include microperimetry, a procedure that assesses retinal sensitivity[42] and visual acuity (VA), which is a measure of the eye's ability to identify shapes and fine details of objects at a given distance.[45] Also, since the macular pigment is localised in the foveal and parafoveal regions of the eye,[39] these regions were structurally assessed in the ENIGMA study by conducting advanced glaucoma module scans which measured macular retina nerve fibre layer thickness (mRNFL), Ganglion cell complex (GCC) and Ganglion cell layer thickness (GCL).[43,44] The assessment of glaucoma-related activity limitation by questionnaire was additionally carried out in the ENIGMA study, using the Glaucoma Activities Limitation 9 questionnaire (GAL 9). This was a perceptual test, designed to subjectively evaluate a patient's ability to perform visually related tasks.[46]

The present study will therefore, furthermore seek to examine any correlation of the visual function, structural and perceptual responses from the ENIGMA study, with the Raman spectroscopic analysis, to further understand their response to macular carotenoids supplementation.

2 Materials and methods

2.1 The ENIGMA study

The ENIGMA study,[39] (registered at ClinicalTrials.gov, identifier NCT04460365) was a randomised, placebo-controlled, double-masked clinical trial, designed to evaluate the macular pigment response of patients with open angle glaucoma (OAG) to supplementation with lutein (10 mg), zeaxanthin (2 mg), and *meso*-zeaxanthin (10 mg) over an 18-month period. The supplement ratio was the same as that of commercially available macular pigment supplementation, Macushield, provided by Thompson & Capper Ltd, Runcorn, United Kingdom. The dose was also deemed safe, with renal, liver, lipid, hematolo-

gic, and inflammatory biomarkers all unaffected by supplementation at these concentrations.[47]

The recruitment was carried out at the Mater Misericordiae University Hospital and Mater Private Hospital (Dublin, Ireland). Study visits were arranged at the Centre for Eye Research Ireland, a dedicated academic clinical trial centre at Technological University Dublin. All necessary research ethical approval were obtained from the Mater Misericordiae Institutional Review Board and Technological University Dublin Research Ethics and Integrity Committee.[39] Written consent forms were supplied by all participants of this study. The study also adhered to the tenets of the Declaration of Helsinki. All experiments were performed in accordance with the European Medicines Agency (EMA) guidelines, and approved by the ethics committee at Technological University Dublin.

62 patients voluntarily participated in the study, of which 42 randomly received the carotenoid supplements, while 20 received the placebo, which contained only sunflower oil. Ocular parameters recorded include macular pigment optical density volume within the central 6° of retinal eccentricity as well as at 0.23°, 0.51°, 0.74°, and 1.02°, recorded using autofluorescence.[48] These eccentricity regions represent the ocular distribution of the MP carotenoids in the fovea of the retina. *meso*-Zeaxanthin is situated most centrally at 0.25° This is followed by zeaxanthin, situated in the 0.5° region and then lutein at 1.0°.[49] Furthermore, visual functional parameters, microperimetry average threshold and visual acuity (VA) were measured alongside structural parameters, macular retina nerve fibre layer thickness (mRNFL), Ganglion cell complex (GCC) and Ganglion cell layer thickness (GCL), which were all measured to assess visual function and glaucoma severity. Lastly, the Glaucoma Activities Limitation 9 questionnaire (GAL 9), which assessed the quality of life of patients, was also analysed as a perceptual parameter.[46]

The main outcome of the trial was a statistically significant increase in MPOD volume (significant time effect: $F(3,111) = 89.31$, mean square error (MSE) = 1656.9; $P < 0.01$), which was observed among those randomised to receive the macular carotenoid supplement over the 18-month trial duration. The study also reported an inverse and statistically significant relationship between baseline MPOD volume and percentage change in MPOD volume over the supplementation period. Notably, the study reported no clinically significant structural or functional changes recorded through the supplementation period.

2.2 Blood samples

Blood samples were collected within the Centre for Eye Research Ireland (CERI) from 62 participants. Non-fasting blood samples were collected by standard venipuncture techniques at each visit and immediately centrifuged for serum. The detailed protocol for processing blood samples have been thoroughly described previously.[49,50] Serum samples were thereafter stored in light-resistant microtubes at 80 °C, until Raman spectroscopic analysis was carried out. When needed, then samples were thawed in a water bath at 37 °C, and Raman spectroscopic measurements were carried out immediately.

Samples obtained at baseline (before supplementation) and at 18 months (after supplementation), were examined only from participants who had received the carotenoid supplements. Samples from participants who received the placebo were not examined, as the original study reported no observable effects.[39]

In total, serum samples from 40 participants of the ENIGMA study were made available for the Raman spectroscopic analysis. 11 of these participants had matching baseline/supplemented samples and were used for the percentage change analysis (Section 3.1). Furthermore, from the 40 participants, data for all clinical parameters analysed were available for only 20 baseline participants samples and 20 eighteen-months supplemented participants samples. 8 of these participants had matching baseline/supplemented samples. These were used for both the combined spectroscopic and regression analysis with baseline and supplemented samples as well as the baseline only/18-month only analysis.

2.3 Raman spectroscopy and pre-processing

Raman spectral measurements[48] were carried out using a Horiba Jobin–Yvon LabRam HR800 spectrometer with a 16-bit Peltier cooled CCD detector, coupled to Olympus 1X71 inverted microscope. A 532 nm laser line of ~12 mW at the sample was used in taking measurements with a 300 lines per mm grating, throughout the study. Serum measurements were performed by focussing the laser into the samples contained in a cover slip glass bottom 96-well plate (Matek), using a ×60 water immersion objective (LUMPlanF1, Olympus).[51] The spectral range employed was 400–4000 cm^{-1} and the back scattered Raman signal was typically accumulated for 5 × 4 seconds. 4–5 spectra were acquired from different spots on each sample.

Pre-processing techniques were then applied to smooth the raw spectra and remove inherent background water and glass contributions before further analysis, as previously described.[52]

2.4 Percentage change of serum carotenoids over supplementation period

The relationship between baseline Raman serum measurements and percentage change in carotenoid supplementation over the 18 month period of the ENIGMA study was estimated for the 11 patients with matching baseline/18 months serum samples, based on the changes in intensity of the Raman signatures of the characteristic carotenoid peaks, specifically at 1004 and 1519 cm^{-1}, before and after supplementation (100 × (18 months − baseline)/baseline). Percentage change was plotted against baseline Raman serum measurement.

2.5. Partial least squares regression analysis (PLSR) and cross validation

Multivariate regression analysis of the Raman responses was carried out following pre-processing, using partial least-squares regression analysis (PLSR) to examine the correlation

of the clinical parameters with the Raman measurements, and explore the predictive capacity of the technique. The PLSR algorithm examines variation in spectral data or predictors, (X matrix), as they relate to the associated factors or responses, (Y matrix), according to the linear equation $Y = XB + E$, where B is the regression coefficient matrix and E is the residual matrix.[53,54] The Y matrix, or "target" variable is usually a quantifiable or systematically varied external factor, in this case measurements from the clinical parameters. It then attempts to maximise the covariance of X (the Raman spectra) and the target, Y, described according to Latent Variables (LV) in a systematic model.[53,54] In summary, it can reduce the number of predictors to a smaller set of uncorrelated components or latent variables which, cumulatively and progressively (LV1 > LV2 *etc*.) account for the co-variance. Least squares regression is therefore carried out on the latent variables, rather than on the original data.[53,55]

The loading of the LV reveals the spectral features which contribute to that LV, and therefore to the co-variance. The Regression co-efficient can be considered the weighted sum of all the contributing LVs, and in spectral analysis, for a good correlation, should yield the spectrum of the constituent components which vary systematically as a function of the target variable.

For this study, PLSR was used to examine the degree of correlation between the ocular parameters measured in the ENIGMA study and the Raman spectroscopic measurements, as represented by the coefficient of determination (R^2). The regression was carried out, first using the combined baseline and supplemented serum from all 20 participants, and then using either baseline or supplemented serum in a set of 20 participants' samples and in a restricted group of 8 matching participants samples. This was done to establish the best correlation from the clinical parameters from the samples made available to the study. The number of LVs used in the regression analysis was chosen by identifying the point at which the cumulative %variance explained reached ~100%.

A Leave-One-Patient Out Cross-Validation (LOPOCV) process,[56] was employed, such that all replicate measurements of a patient were grouped and simultaneously removed from the training, to ensure that measurements of the same patient are not used to both train and test the PLSR model. Ultimately, the PLSR model constructed can be employed as a predictive model, which can be used, for example, to predict the value of the target variable, based on the spectrum of an unknown sample, or *vice versa*, with an accuracy described by the Root Mean Squared Error of Prediction (RMSEP).[56,57]

3 Results

3.1 Raman spectroscopic analysis: percentage change over 18 months

An example of a typical Raman spectrum of carotenoids (beta carotene), obtained at 532 nm is shown in Fig. 1(a), highlighting the three characteristic Raman features of carotenoids at 1004, 1158 and 1519 cm^{-1}. The carotenoid bands are associated with carbon–carbon single and double bond stretches of the polyene backbone and methyl bends of the carotenoid structure.[58] The laser wavelength is close to the optical absorption resonance of the carotenoids, and so these features are seen to dominate the spectra of the patient serum samples,[36,59] which also show characteristic features of the serum proteins, for example the amide I band at ~1640 cm^{-1}.[60] Raw Raman spectra of body fluids contain many other background materials that can obscure the features of interest in the spectra, in this case, serum carotenoids. Background correction was therefore carried out to remove large features like water and glass and to minimise the noisiness of the spectra.

The relationship between baseline Raman serum measurements and percentage change in Raman intensity of two carotenoid peaks 1004 and 1519 cm^{-1} over the 18 month period of the ENIGMA study is represented individually in Fig. 1(b) and (c), the dashed line being a guide to the eye. Both scatter plots show inverse relationships between baseline serum and the percentage change over the 18-month supplementation period. Overall, an increase in the Raman intensity of the serum carotenoid features is observed after supplementation across most participants in the supplemented set, the lowest baseline participants showing the greatest change over 18 months and the highest baseline participants showing little or no change over 18 months. This result is consistent with the observations of the ENIGMA study,[39] and is suggestive of the validity of a correlation of blood serum measurements of carotenoid content with ocular health.

3.2 Raman spectroscopic analysis of patient serum: an example of multivariate regression with MPOD (0.23°)

As an illustration of the methodology applied to explore the potential correlations between the ocular parameters and the Raman spectroscopic data, Fig. 2 shows the details of the PLSR analysis for the example of MPOD 0.23°. Fig. 2(a) illustrates the cumulative %variance explained as a function of the number of PLS components (LVs) in the PLSR of the spectral data for the 8 matched baseline/supplemented participants set against the MPOD 0.23° values, indicating that 4 LVs are sufficient to model the variance, avoiding overfitting. The resultant analysis showed a good degree of linearity ($R^2 = 0.92$) between the fitted (predicted) and observed (measured) responses, corresponding to the input target values for MPOD 0.23°, as shown in Fig. 2(b). Fig. 2(c) shows the spectrum of the PLSR loading of the first LV (~48% of the explained variance), while Fig. 2(d) is the PLSR regression coefficient. Both figures show features which are characteristic of the carotenoid structure, therefore highlighting the high degree of correlation between the spectroscopic responses and the clinical parameter. Fig. 2(e) shows the dependence of the Mean Squared Error of Prediction (MSEP) on the number of LVs used, which is seen to minimise after 3 LVs.

In summary, the PLSR analysis indicates that the MPOD 0.23° measurements of the patient cohort are well correlated

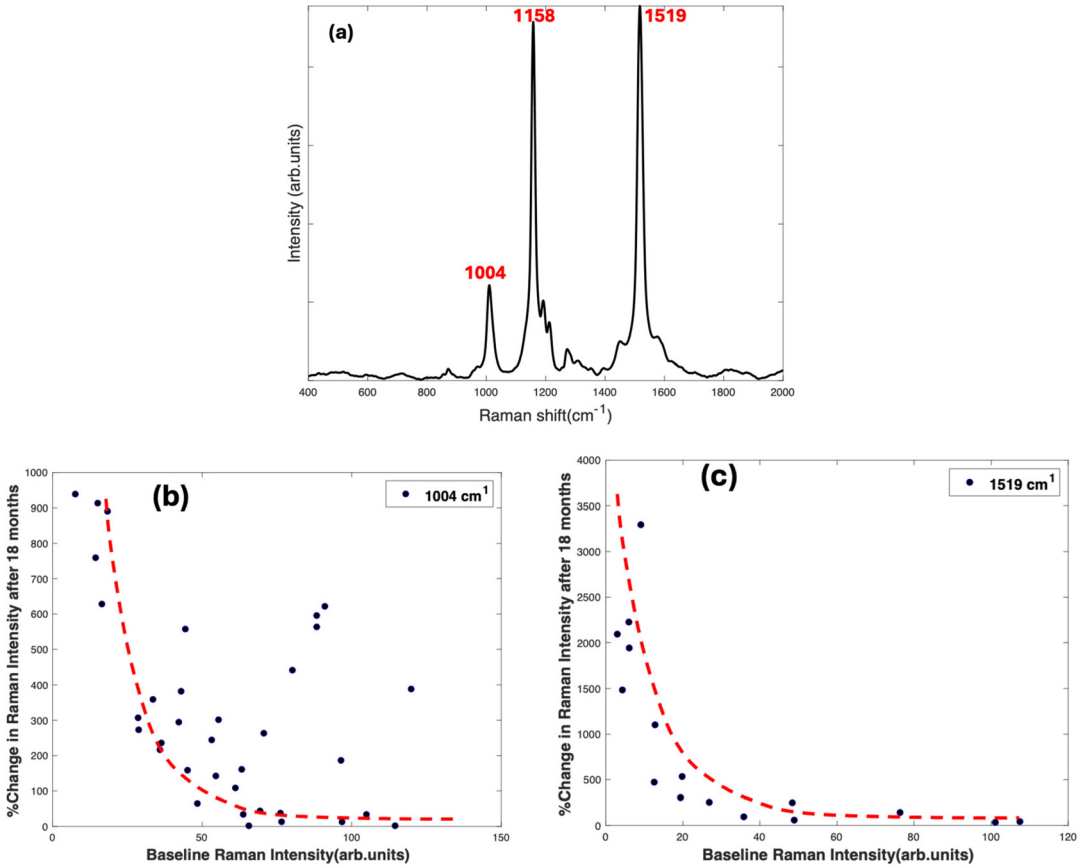

Fig. 1 (a): Typical Raman spectrum of carotenoids (Beta carotene), obtained at 532 nm using ×60 objective and showing characteristic bands at 1004, 1158 and 1519 cm^{-1}. (b) Percentage change of Raman intensity of serum carotenoids after 18 months of carotenoid supplementation as a function of baseline serum Raman intensity, based on the 1004 cm^{-1} carotenoid peak and (c) based on 1519 cm^{-1} carotenoid peak. The dashed lines are guides to the eye.

with the Raman spectral profile of the blood serum (R^2 = 0.92), and that the clinical parameter can be predicted from the PLSR model with an accuracy represented by RMSEP = 0.21. The percentage error of RMSEP compared to the mean of the range of measurements for all participants was also estimated as (RMSEP/mean) × 100 = 35.2%.

3.2 Raman spectroscopic analysis: correlation with clinical parameters

PLSR analysis was carried out on the patient serum Raman spectroscopic data against the individual clinical parameters from the ENIGMA study to explore correlations between the ocular performance and the supplementation responses, as measured using Raman spectroscopic analysis of the patient blood serum samples, as described in Section 3.1. The analysis was carried out against MPOD, which is an estimation of the carotenoids in the macula[31] as well as the other structural, functional and perceptual parameters. The results of the regression analysis of the different serum treatment groups with MPOD are presented in Table 1. The results of the analysis of co-variance of the spectroscopic results with MPOD within the central 6° of retinal eccentricity is shown, as well as those of 0.23°, 0.51°, 0.74°, and 1.02°. The analysis was performed on the whole dataset (baseline + 18 months), and on the 20 patient and 8 patient subsets. The table lists the R^2, RMSEP and mean values for the MPOD analysis. It also shows the percentage error, which was calculated by dividing the RMSEP by the mean value of the respective parameter. The results generally show reasonable correlations with all MP eccentricity regions and in the different patient groupings. The combined regression analysis showed correlations with R^2 values between 0.94–0.97 and reasonably low values of RMSEP, although with high percentage errors (between 48–57%). The analysis improved in the regression with baseline samples, when only 8 participants samples were used, resulting in R^2 values ranging from 0.80–0.96 and an error range of 45–49%. However, when regressed with 20 baseline serum samples, lower R^2 values were obtained (0.67–0.95) as well as higher percentage errors (49–71%). In the analysis with 18 months supplemented samples, further improvements in the regression analysis were achieved using both 8 patients (R^2 = 0.62–0.92, %RMSE = 29–44%) and 20 participants (R^2 = 0.98–0.99, %RMSE = 46–49%).

Overall, the best correlations with MPOD were obtained using the supplemented samples, as the regression yielded the

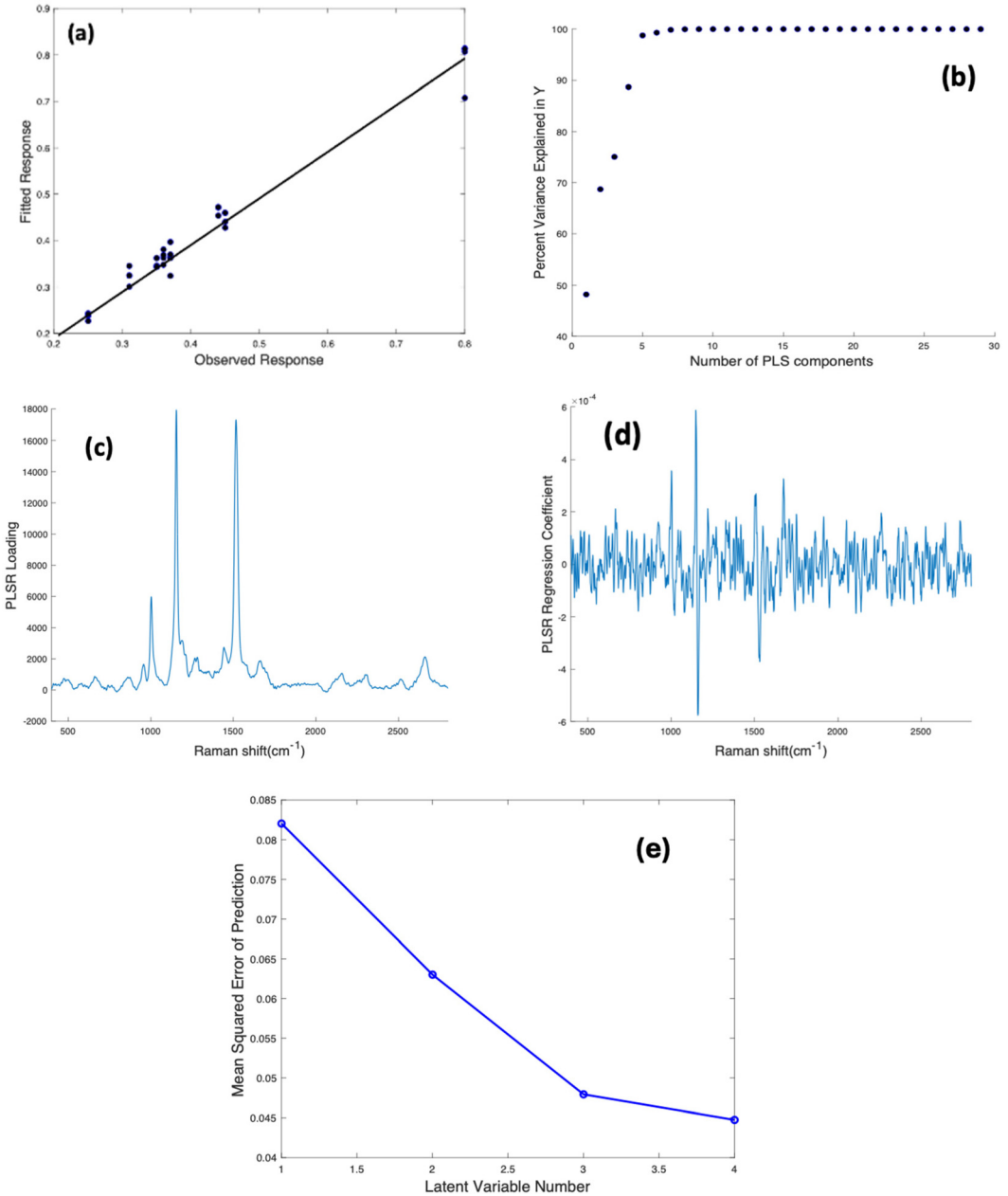

Fig. 2 (a): PLSR analysis of correlation of patient blood serum Raman spectra *versus* MPOD 0.23° measurements. (b): Cumulative percentage variance of PLSR of participants blood serum Raman spectra *versus* MPOD 0.23° measurements. (c): PLSR loading of blood serum, showing the spectrum of first latent variable dominating the regression coefficient. (d): PLSR regression coefficient of participants blood serum Raman spectra *versus* MPOD 0.23° measurements, constructed using 6 latent variables. (e): Estimated mean squared cross-validation error *versus* number of components (latent variables) for MPOD 0.23°.

lowest errors with the 8 participants only group (<44%) and the best (highest) R^2 with the 20 participants' group (>0.97). Notably, with the supplemented 8 participants serum analysis, the regression appeared to improve as the eccentricity region narrowed, from MPOD 6° to 0.23°, yielding slightly better RMSEP (0.15–0.21) and lower percentage errors (<36%) at 0.23 and 0.51, compared to MPOD at 6°, 1.02° and 0.74° (RMSEP = 0.20–0.22, %RMSE > 42%).

In Table 2, the results of regression analysis against the visual functional parameters, Microperimetry and visual acuity (VA) recorded during the ENIGMA study are shown. These parameters were also analysed against the different treatment groups, similar to Table 1 and the R^2, RMSEP, mean value and percentage error were reported. Microperimetry showed good correlation across the serum subsets, with R^2 of 0.88–0.99 and %RMSE < 34%, except for a much higher error (40%) obtained

Table 1 Multivariate regression analysis of patient serum with macular pigment optical density

Variable	Baseline + 18 months	Baseline (8 participants)	Baseline (20 participants)	18 months (8 participants)	18 months (20 participants)
MPOD 6					
R^2	0.94	0.96	0.70	0.77	0.99
RMSEP	3758.20	2859.90	3914.80	3769.10	3646.90
Mean	6484.40	6075.95	5510.27	8957.48	7373.82
%RMSE	57.95	46.90	71.04	42.07	49.45
MPOD 1.02					
R^2	0.97	0.95	0.67	0.62	0.99
RMSEP	0.15	0.17	0.219	0.204	0.19
Mean	0.20	0.37	0.34	0.47	0.43
%RMSE	51.0	46.0	64.26	43.57	45.88
MPOD 0.74					
R^2	0.95	0.96	0.65	0.75	0.99
RMSEP	0.21	0.18	0.224	0.221	0.224
Mean	0.44	0.42	0.39	0.52	0.48
%RMSE	48.0	44.97	57.51	42.48	46.72
MPOD 0.51					
R^2	0.95	0.95	0.95	0.85	0.98
RMSEP	0.23	0.18	0.222	0.15	0.23
Mean	0.45	0.42	0.40	0.52	0.50
%RMSE	52.02	44.78	55.67	29.40	47.50
MPOD 0.23					
R^2	0.95	0.80	0.96	0.92	0.99
RMSEP	0.26	0.23	0.23	0.21	0.26
Mean	0.51	0.48	0.46	0.60	0.55
%RMSE	52.62	48.54	49.30	35.25	47.50

Table 2 Multivariate regression analysis of patient serum with ocular function clinical parameters

Variable	Baseline + 18 months	Baseline (8 participants)	Baseline (20 participants)	18 months (8 participants)	18 months (20 participants)
Microperimetry					
R^2	0.93	0.99	0.91	0.88	0.94
RMSEP	7.59	5.62	8.59	6.40	5.49
Mean	22.45	23.62	21.38	23.75	23.43
%RMSE	33.81	23.80	40.17	26.95	23.41
Visual acuity					
R^2	0.96	0.99	0.99	0.91	0.91
RMSEP	9.37	3.22	11.02	9.15	8.56
Mean	95.26	94.03	93.90	98.87	96.61
%RMSE	9.83	3.42	11.74	9.25	8.86

with the 20 patients-baseline serum set. VA, on the other hand, showed a consistently good correlation across the serum sub sets, yielding R^2 values > 0.90 and %RMSE < 12%. For both functional parameters, similar to the MPOD analysis, the weakest correlation was observed from the baseline regression with 20 participants.

The results of the regression analysis with the ocular structural parameters, Ganglion cell complex (GCC) thickness, Ganglion cell layer (GCL) thickness and macular retinal nerve fibre layer (mRNFL) thickness are presented in Table 3. The analysis, carried out in the same way as in Tables 1 and 2, shows relatively good correlation for the three parameters across the regression serum subsets (R^2 = 0.75–0.98; %RMSE < 30%). The best performance is seen in the regression with the 8 patients supplemented serum set (%RMSE < 23%), except for GCL, which performed best with the 20 patients supplemented serum set. The scores appear to weaken with the 20 patients supplemented and baseline sets and is weakest with the combined serum baseline and supplemented serum set, especially for GCC6 and mRNFL (R^2 = 0.84–0.93, %RMSE > 18%). GCC performed weakest with the 8 patients supplemented set (R^2 = 0.75, %RMSE = 29%). Overall, GCC had the strongest correlation across the structural parameters with percentage error as low as 4.63% (8 patient supplemented serum group) and R^2 up to 0.97 (20 patients baseline group).

Lastly, the results of the regression analysis with the ocular perceptual parameter analysed in the ENIGMA study is reported in Table 4. The Glaucoma Activities Limitation 9 (GAL 9) questionnaire was a quality-of-life questionnaire administered during the ENIGMA study. The regression analysis against GAL 9 showed a high degree of correlation across the baseline, supplemented and the combined serum subsets (R^2 > 0.91). The weakest correlation was seen with the baseline only analysis with 20 patients, which produced the highest

Table 3 Multivariate regression analysis of patient serum with ocular structural parameters

Variable	Baseline + 18 months	Baseline (8 participants)	Baseline (20 participants)	18 months (8 participants)	18 months (20 participants)
GCC thickness (μm)					
R^2	0.85	0.83	0.97	0.82	0.94
RMSEP	13.90	11.61	13.16	3.40	12.69
Mean	75.56	76.87	75.86	73.49	75.29
%RMSE	18.39	15.11	17.34	4.63	16.85
GCL thickness (μm)					
R^2	0.94	0.98	0.95	0.85	0.99
RMSEP	5.48	4.95	4.22	9.02	4.47
Mean	26.11	26.68	25.81	25.54	26.42
%RMSE	20.98	18.54	16.36	35.32	16.90
mRNFL thickness (μm)					
R^2	0.94	0.98	0.94	0.94	0.93
RMSEP	6.63	6.78	7.16	5.29	6.70
Mean	24.22	24.84	24.86	23.65	23.63
%RMSE	27.37	27.29	28.80	22.4	28.33

Table 4 Multivariate regression analysis of patient serum with GAL 9, a perceptual parameter from the ENIGMA study

Variable	Baseline + 18 months	Baseline (8 participants)	Baseline (20 participants)	18 months (8 participants)	18 months (20 participants)
GAL 9					
R^2	0.92	0.99	0.94	0.93	0.93
RMSEP	8.15	6.46	9.97	6.68	7.36
Mean	14.24	14.29	15.10	13.58	13.39
%RMSE	57.26	45.19	66.01	41.17	54.94

error (66%) while the strongest correlation was with the 18 months analysis with 8 patients (R^2 = 0.93, %RMSE = 41.17%).

3 Discussion

Raman spectroscopy continues to be explored as a reliable tool for clinical diagnosis, as well as for understanding disease progression.[61–63] Previous studies have demonstrated the robustness of the technique for carotenoid measurements in skin, as an indicator of nutritional status, and in living human eyes, in which the carotenoid contents are associated with ocular health.[1,28] However, as blood is the prime specimen of interest in clinical diagnosis and contains all dietary carotenoids,[51,64] it is important to establish the competency of the method to measure carotenoids directly in the blood, as this would provide and validate a more accurate measure of dietary carotenoids generally. More specifically, it can provide a suitable alternative way of monitoring MP carotenoids in clinics, especially as the current methods are not widely accessible.

Previously, the methodology to monitor and analyse changes in the composition of blood carotenoids using Raman spectroscopy was demonstrated using baseline and supplemented serum samples from the ENIGMA study.[38] In the present study, the results of the percentage change for the supplementation period further validates the method as a reliable potential quantitative tool for analysing serum carotenoids. The inverse relationship observed between baseline Raman serum measurements and percentage change in Raman intensity over the 18 month period of the ENIGMA study is similar to that reported in the ENIGMA study. This important finding can guide supplementation strategies in the future as it implies greater relative benefit of the supplementation for participants with low MPOD/serum carotenoids before supplementation. Interestingly, it is also suggestive of a plateauing effect for higher doses and prolonged intake of the carotenoid supplementation, as previously suggested in literature.[65]

Evaluation of the correlation of serum Raman measurements with MPOD and other clinical parameters measured from the ENIGMA trial was carried out to explore the predictive capacity for MP status based on Raman spectroscopy of serum. The use of resonant or near resonant laser wavelength sources is a huge advantage to the Raman spectroscopic analysis of carotenoids, as it clearly enhances the carotenoid Raman features as seen in this study. The PLSR technique employed also clearly demonstrates how patient ocular health can be assessed on the basis of the Raman spectrum of blood serum. For instance, in the example illustrated, a good correlation between MPOD 0.23° and the spectral measurements highlights the potential of the method to predict MPOD 0.23° measurements.

The regression analysis was carried out using both treatment groups (baseline and 18 months supplemented serum), which were made up of subsets of 8 and 20 participant samples, in order to establish the strongest correlations with

the ocular measurements. Also, because of the relatively small patient sample size, the application of a rigorous cross validation process was warranted. The LOPOCV employed was to avoid possible underestimation or overestimation of the regression analysis.

Generally, the correlation with the ocular parameters measurements was best highlighted in the analysis with the supplemented participants serum, especially when carried out with only 8 participants. This was consistent across the MPOD measurements as well with the structural, functional and perceptual parameters. However, some reasonable correlations were also established across the other sets of patient serum (combined, supplemented serum and baseline only). The worst performance was with the 20 participants baseline serum. A greater degree of variance in the baseline data can result in such relatively poor performance, whereas a controlled supplementation regimen reduces this variance in the supplemented set.

Specifically, the correlation of the Raman serum measurements with MPOD measurements produced reasonably good linearity across the different retinal eccentricity regions measured. The variance in the data set could have been caused by a number of factors, including demographic characteristics, dietary lutein and zeaxanthin intake, serum cholesterol and lifestyle factors like smoking and exercise.[66,67] The concentration of other dominant carotenoids *e.g.* Beta carotene and lycopene can also markedly impact on the variance in the data set. In future, controlling for these factors can minimize the variance. This can be done for instance, by conducting detailed surveys to collect comprehensive data on demographic factors (age, gender, socioeconomic status, race/ethnicity), lifestyle factors (smoking habits, alcohol intake, physical activity levels), and diet (*e.g.*, food frequency questionnaires). Patients medical history *e.g.* serum cholesterol levels could also be measured to account for differences in lipid metabolism, which can influence carotenoid levels.[41] Data from the various cofounding factors can then be adjusted for in the regression analysis.

To obtain an overall and accurate estimation of the MP volume, in the ENIGMA study, measurements were recorded across the central 6° but also across smaller retinal eccentricities (using the autofluorescence technique). This was done in order to make accurate reference to previously published gold standard methods of measuring MPOD.[68] From the Raman spectroscopic analysis, the correlation with MPOD appears to improve slightly as the eccentricity regions narrowed. For instance, for the 8 supplemented participants set, at 0.23° and 0.51°, the error margins were lowest compared to the larger regions measured. Remembering the ocular distribution of the MP carotenoids, *meso*-zeaxanthin is situated most centrally within the 0.25° region, zeaxanthin within 0.5° and lutein within 1.0°.[49] The supplementation, which contained a 10 : 2 : 10 ratio of lutein, zeaxanthin and *meso*-zeaxanthin, was formulated to boost the central MP region, as studies have demonstrated the addition of *meso*-zeaxanthin to supplementation can result in increased overall serum MP distribution and improved visual function.[49] The observed improvements in the acuity regions which are most central to the macular pigment is consistent with them having received the highest boost from the supplementation. The increased correlation of the Raman serum measurements with the narrowed field of acuity is hence consistent with the supplementation regimen.

The correlation of MPOD with Raman serum carotenoid measurements is an advancement in Raman spectroscopy of dietary carotenoids and has very good implications for monitoring macular pigment status and ocular health in general. Serum levels of carotenoids are commonly used as biomarkers for determining ocular carotenoid status,[69] but the standard method employed, HPLC is tedious and expensive.[69,70] To date, the correlations of Raman spectroscopy of serum carotenoid concentrations and MPOD have not been reported. However, there have been significant correlations between autofluorescence and HFP measurement of MPOD with skin Resonance Raman spectroscopy (RRS), in various studies.[27,71,72] There have also been significant correlations with serum HPLC carotenoid measurements.[72–74]

In addition to MPOD, the other clinical factors measured showed varied but mostly strong correlations with the Raman spectroscopic serum measurements, even though their variation was not deemed to be clinically meaningful in the ENIGMA study. The functional factors were generally well correlated with serum measures in the supplemented 8 participants subset. The structural parameters were also well correlated across the serum subsets. The perceptual response measured performed similarly to the MPOD measurements, as it showed a higher degree of correlation. The correlation of the Raman analysis with the functional, structural and perceptual ocular responses strongly support the MPOD correlation and is an interesting finding that warrants further investigation in the future.

Notably, although the ENIGMA trial monitored participants at 6 month intervals, only serum samples at the 18 month stage were made available for the Raman spectroscopic study. In the ENIGMA trial, macular pigment levels continued to increase throughout the full 18-months, indicating a benefit of long-term supplementation. Stratified analysis have shown that the augmentation of the macular pigment was most effective when supplementing with the MP carotenoids during trials lasting longer than 12 months.[75,76] Future intervention trials certainly should prioritize an appropriately powered study with longer timeframes (for example, 2- to 5-year treatment window) to explore the potential extended benefits of these potent antioxidant and anti-inflammatory nutrients for visual function and ocular health in glaucoma, with 6 monthly ocular health monitoring, and parallel Raman monitoring of the evolution of the serum content.

Overall, the implications of establishing a reliable quantitative method for analysis of serum carotenoids are potentially far reaching. It can advance the screening of major MP related diseases like AMD and glaucoma, and also lead to a better understanding of disorders which might be directly or indirectly associated with MP and serum carotenoid levels. For instance, studies have also shown the MP carotenoids play a

protective role in the retinal pigment epithelium of neonates and new-born infants.[77–79] They are also involved through the maturational stage and childhood development.[80] Furthermore, MP carotenoid levels have also been strongly linked with cognitive function and the progression of diseases like Alzheimer's and mild cognitive impairment.[50]

4 Conclusion

The findings from this study strongly support those from the previously published ENIGMA study and generally point towards strategies for guiding carotenoid supplementation regimen based on baseline carotenoid level. Additionally, the correlation of Raman spectroscopy of serum with MPOD primarily presents the method as a promising, easy and reliable alternative for estimating MP carotenoids *in vivo*. The findings can therefore potentially increase the understanding of other associated MP disorders. Finally, the methodology could potentially be applied extensively in the diagnosis and management of MP related diseases like AMD and glaucoma, especially in circumstances where MPOD measurement techniques are inaccessible or simply unsuitable for the participants.

Author contributions

Conceptualization, J. U., E. L., J. L. and H. J. B.; methodology, J. U. and H. J. B.; formal analysis, J. U. and H. J. B.; investigation, J. U., E. L.; resources, H. J. B.; data curation, J. U.; writing – original draft preparation, J. U. and H. J. B.; writing – review and editing, J. U., E. L., J. L. and H. J. B.; supervision, E. L., J. L. and H. J. B.; funding acquisition, H. J. B.

Human subjects

Human subjects were included in this study. The human ethics committees at Mater Misericordiae and the Technological University Dublin approved the study. All research adhered to the tenets of the Declaration of Helsinki. All participants provided informed consent. No animal subjects were included in this study.

Data availability

The data that support the findings of this study are openly available from the Mendely database, available at: **https://data.mendeley.com/datasets/8pwpbcrjd8/1**, **https://doi.org/10.17632/8pwpbcrjd8.1**

Conflicts of interest

The authors declare no conflict of interest.

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