Association of Human Plasma Metabolomics With Delayed Dark Adaptation in Age-Related Macular Degeneration

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Student Name: Janice Kim, BS

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Mentor Name(s) and Affiliations: Deeba Husain, MD, Vitreoretinal Service, Massachusetts Eye and Ear

Collaborators, with Affiliations: Wonil Chung, PhD, Harvard T.H. Chan School of Public Health, Liming Liang, PhD, Harvard T.H. Chan School of Public Health, Inês Laínes, MD, Massachusetts Eye and Ear

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Janice Kim, Wonil Chung, Inés Laínes, Liming Liang, Deeba Husain

PURPOSE: To assess the association between plasma metabolomic profiles and dark adaptation (DA) in all stages of age-related macular degeneration (AMD).

METHODS: 56 AMD patients (14 early-stage AMD, 31 intermediate-stage AMD, 11 late-stage AMD) and 19 control patients without any vitreoretinal disease were prospectively recruited from January 2015 to June 2016. Participants underwent profiling for metabolomes in fasting blood and urine samples through the use of mass spectrometry (MS). Nontargeted MS analysis was performed by Metabolon, Inc., using ultrahigh-performance liquid chromatography tandem MS. For biological interpretation, pathway enrichment analysis of significant metabolites was performed using MetaboAnalyst. Subjects also obtained DA testing in both eyes with the AdaptDx (MacuLogix, Middletown, PA) DA extended protocol (20 minutes). Multivariate logistical regressions were performed using various metabolites as predictors for dark adaptation in AMD patients versus controls, while controlling for age.

RESULTS: Multivariate logistical regressions between time to dark adapt (continuous variable, truncated at 20 minutes, limit of the test) and 544 general metabolites identified 1 significant metabolite, alpha-tocopherol (AT), as different between AMD patients and control patients.

CONCLUSIONS: Participants with AMD may have altered levels of alpha tocopherol compared with controls that could potentially predict ability to dark adapt and supports oxidative stress as a pathogenic mechanism for AMD.

Table of Contents

Glossary of Abbreviations:

DA: dark adaptation
AMD: age-related macular degeneration
RPE: retinal pigment epithelium
DHA: docosahexaenoic acid
ROS: reactive oxygen species
MEE: Massachusetts Eye and Ear
MS: mass spectrometry
RIT: rod intercept time (in minutes)
FDR: false discovery rate
AIC: Akaike information criterion
AT: alpha-tocopherol
IQR: interquartile range (in minutes)
BCVA: best-corrected visual acuity
IOP: intraocular pressure
logMAR: logarithm of the minimal angle of resolution
OD: right eye
OS: left eye

Introduction:

Age-related macular degeneration (AMD) is a leading cause of blindness among subjects 50 years of age or older. Currently, our understanding of the mechanisms of macular degeneration is complex. Inflammation is one proposed pathogenic mechanism for AMD—drusen, a hallmark of AMD, is composed of many pro-inflammatory factors such as apolipoprotein E, acute phase proteins, and IgG and dry AMD is associated with the activation of resident immune cells in the retina and choroid.[1–32] Genetic analyses of AMD patients also demonstrate the presence of genetic variations encoding components of the alternative complement activation pathway.[1] Additionally, growing evidence suggests oxidative stress as important to the pathophysiology of AMD. Smoking, which is associated with oxidative damage, is a main risk factor for AMD. Constant exposure to light and the oxidative stress occurring in tissues results in lipid peroxidation; AMD is associated with the accumulation of advanced lipid peroxidation end products that lead to the apoptosis of photoreceptors and retinal pigment epithelial (RPE) cells.[1,4,5] AMD pathobiology can be further elucidated through the use of metabolomics, the qualitative and quantitative analysis of metabolites.[6] Metabolomics is the study of metabolites, which are low molecular weight molecules that are generated downstream of all genetic transcription and translation processes as well as in response to external stimuli.[7–9] The metabolome is most closely related to phenotype and can provide insights into the interactions between genetics, environment, and lifestyle that contribute to multifactorial diseases such as AMD. A previous study had investigated the role of plasma metabolomics to a single subtype of AMD (choroidal neovascularization), but Dr. Husain's group was the first known to assess plasma metabolomics across all stages of AMD.[7] Her results revealed that AMD patients have distinct metabolic profiles compared to those without AMD, particularly in metabolites related to glycerophospholipid metabolism.[7] Prior literature supports altered lipid metabolism as a potential pathogenic mechanism in AMD.[1,3] Several genetic mutations in cellular lipid transport, for example ATP-binding cassette transporters, have been shown to play critical roles in the pathogenesis of atherosclerosis, AMD, and Alzheimer's disease.[3,10]

Despite a relative understanding of the potential pathogenic mechanisms for AMD, very limited options are currently available to assess for and halt the progression of early AMD to its blinding stages. Only the AREDS formulation has been shown to decrease the rate of progression, but to a limited extent and solely in patients with intermediate AMD.[11] While studies looking at antioxidant-rich diets and possible therapeutic strategies to alleviate inflammation, prevent oxidative damage, and reduce accumulation of retinal oxides are underway, these are still currently undergoing evaluation, with mixed results. [1,12] In terms of assessing for AMD progression, drusen is often a hallmark sign of early AMD; however, not all individuals with drusen develop AMD and not all drusen is related to age-related macular degeneration.[13] Furthermore, studies have shown that drusen quantity did not correlate with disease severity or functional outcomes such as dark adaptation, making it difficult to know if and when early AMD patients will progress based on visual exam alone.[13–15] Visual acuity currently remains the gold-standard outcome to assess for visual function in AMD, even though patients usually do not present with vision loss until late into the disease.[15] Besides the characteristic loss of central vision, AMD can also cause many other symptoms such as blurred and distorted vision, extra sensitivity to glare, and difficulty reading in low light with eventual vision loss. Other functional outcome measures have been studied to assess for progression of vision loss in AMD, such as contrast sensitivity, low-luminance visual acuity, photopic or scotopic light sensitivity, and dark adaptation (DA).[15,16] DA, in particular, seems promising, as early stage patients often report loss of night vision and reduced ability to adapt from brightly lit to dark environments, even in the setting of normal visual acuity.[17] Recent studies have shown that, with the use of a commericially available device—the AdaptDx (MacuLogix, Middletown, PA) dark adaptometer—dark adaptation can differentiate between patients with and without AMD, and across severity stages of disease.[15,16,18,19]

The mechanisms behind why AMD is associated with DA changes remain partially understood. Following exposure to intense illumination, the ability to recover visual function to normal, otherwise known as “dark adaptation,” depends on the visual cycle.[20] The visual cycle requires coordinated activity between the photoreceptors

present in rods and the RPE to recycle rhodopsin, the photopigment that enables vision in low light. AMD patients may have decreased dark adaptation ability due to the loss of docosahexaenoic acid (DHA), resulting in decreased binding affinity of interphotoreceptor retinoid binding protein to intermediate substrates necessary for photopigment regeneration.[21,22] Intrinsic abnormalities of the RPE layer itself can also slow delivery of retinoid intermediate to the RPE due to the excessive accumulation of lipofuscin.[22–24] Lamb and Pugh hypothesized that AMD changes such as the thickening of Bruch's membrane and deposition of hydrophobic lipids may also hinder transport of vitamin A from choroidal circulation, resulting in a vitamin A deficient RPE/retina that then reduces the amount of 11-cis-retinal (a vitamin A derivative) available to combine with opsin to form rhodopsin.[24,25] Further increasing our complete understanding on the mechanisms behind visual impairment in AMD is crucial to clarify both the complex pathophysiology driving AMD and address the great unmet need for potential markers and treatments for AMD progression.

Our group will be the first to study the association between metabolic profiles of AMD subjects and dark adaptation. By assessing the association between metabolic profiles and time to dark adapt, this project will help us understand the mechanisms behind why DA changes in AMD. This may also increase our current knowledge on the mechanisms involved in the pathophysiology of AMD, which may eventually point to new druggable targets for the early/ intermediate forms of AMD. Our aim is to assess the association between plasma metabolomic profiles and time to dark adapt, with the ultimate goal of increasing our understanding behind the visual impairment and pathogenic mechanisms in AMD.

Student Role:

For this project, my role included data collection (recruiting patients and controls from Massachussetts Eye and Ear (MEE) clinics, obtaining and processing blood and urine samples to analyze for metabolites) and data analysis. I worked closely under the supervision of Wonil Chung, PhD, and Liming Liang, PhD, who are statisticians at the Harvard T.H. Chan School of Public Health. Under the supervision of our collaborators, I explored various methods of multivariate logistical regressions for testing on the dataset. I also attended monthly AMD meetings and statistician meetings to troubleshoot any issues surrounding the data analysis and creation of best-fit models that would identify significant metabolites while controlling for all possible confounders.

Methods:

Study design:

This study is derived from a cross-sectional, observational study performed by the Department of Ophthalmology at MEE, Harvard Medical School, Boston, United States. From January 2015 to June 2016, 75 total participants were prospectively recruited, 56 of whom were AMD patients (14 early stage AMD, 31 intermediate stage AMD, and 11 late stage AMD). We excluded subjects with any other vitreoretinal disease, active uveitis or ocular infection, significant media opacities that precluded the observation of the ocular fundus, refractive error equal to or greater than 6 diopters of spherical equivalent, formal diagnosis of glaucoma with a cup-to-disc ratio superior to 0.7, history of retinal surgery, history of any ocular surgery or intraocular procedure (such as laser and intraocular injections) within the 90 days before enrollment, and diagnosis of diabetes mellitus, with or without concomitant diabetic retinopathy. 19 controls with no evidence of AMD and aged >50 years were also recruited, and the same exclusion criteria were applied. Patients were profiled for metabolomes through fasting blood and urine samples through the use of mass spectrometry (MS). Nontargeted MS analysis was performed by Metabolon, Inc., using ultrahigh-performance liquid chromatography tandem MS. Subjects also underwent DA testing

using the AdaptDx dark adaptometer (Macu- Logix, Middletown, PA) where eyes were bleached by exposure to a 505-nm photoflash (0.8-ms duration, 1.8x104 scot cd/m2 intensity) and sensitivity measurements were measured afterwards by having the subject focus on a fixation light and indicate when a stimulus light was visible by pushing a hand-held button.

Statistical and Data Analysis:

Our primary analysis looked at time to dark adapt (continuous variable, truncated at 20 minutes, limit of the test) as the primary outcome and utilized multiple logistic regressions with different plasma metabolites as predictors. Multivariate analyses accounted for confounding factors, including age. A secondary outcome considered the rod-intercept time (RIT), which was the total time to dark adapt to a certain threshold of light intensity. Best-fit models were validated with the Akaike information criterion (AIC) and isolated metabolites were assessed for significance by adjusted *P*-values <0.05, based on False Discovery Rate (FDR). Errors from multiple hypothesis testing was accounted for by the Bonferroni correction. For biological interpretation, the significant metabolites were assessed on pathway analysis using MetaboAnalyst 3.0.

In terms of discerning the correct sample size for our study, there was no preliminary data to enable power calculations. Power calculations for metabolomic studies in general are complex and require specific tools.[6] However, the national guidelines for human studies in metabolomics suggest at least 20-30 patients per group in general, which was met with this proposal including 56 AMD patients and 19 controls.[26] In addition, statistical power was increased by analyzing dark adaptation as a continuous variable in relation to time, which increased the data set from 75 individual observations to over 2,000 time-point observations (when accounting for time point data for each patient). In any case, this is a pilot study (the first to look at dark adaptation and its association with metabolomics and AMD), and it will be crucial for generating data to help with robust power calculations in the future.

Results:

A total of 75 participants were prospectively recruited for the study. 56 participants were clinically diagnosed with AMD and subcategorized as early (n=14, 25%), intermediate (n=31, 55%), and late (n=11, 19.6%). 19 controls (25.3%) over the age of 50 without AMD were recruited. Table 1 represents the clinical and demographic characteristics of the study population. Dark adaptation was tested in both eyes, if possible, for each participant and the RIT was calculated for each subgroup, as shown in table 2. The RIT was longest in patients with intermediate AMD (15.3 min) and shortest in controls (5.1 min).

In both cohorts, 605 metabolites were measured. Of these, 544 metabolites were identified as endogenous metabolites based on prior research by the lab. To assess for possible associations between the 544 metabolites and dark adaptation times in each cohort, we conducted a series of logistic regression analyses accounting for potential confounders (age) and FDR. Many models were attempted by varying fixed and random effect terms to try and find one that best fit the data. Overall, the workflow analyses can be simplified into 3 different models.

The first model looked at the dark adaptation times with RIT as a fixed time-point outcome for both eyes in each patient. A general linear model associated RIT with the various 544 metabolites, controlling for age as a confounder. These results revealed that 19 metabolites differed significantly (p-value < 0.05; q-value < 0.05, table 3) between AMD patients and controls. As shown, most of the significant metabolites (q-value) were lipids (n = 6; 31.6%) and amino acids (n = 6, 31.6%), followed by cofactors and vitamins (n = 4, 21.1%), nucleotides (n = 2, 10.5%), and carbohydrates (n = 1; 5.3%).

The second model utilized continuous time data for each patient in each eye in order to increase the power of the sample size. A linear mixed-effects model was implemented and accounted for random effect parameters related to eye laterality and the individual subject. The linear mixed-effects model demonstrated 2 significant

metabolites (p-value < 0.05; q-value < 0.05, table 4) between AMD patients and controls: alpha-tocopherol (cofactor and vitamin) and glycerate (carbohydrate). These metabolites were also part of the 19 significant metabolites found by the generalized linear model.

The third model was a linear mixed-effects model accounting for autoregression using corAR1, which is generally good for temporal data. The linear mixed-effects model with autoregression demonstrated 1 significant metabolite (p-value < 0.05; q-value < 0.05, table 5) between AMD patients and controls: alpha-tocopherol (cofactor and vitamin). This model is likely the best-fit model since the AIC was also the lowest for this model (AIC= 35.5).

The top 20 metabolites—involved in amino acid, carbohydrate, lipid, and nucleotide pathways—that differed significantly between AMD patients and controls in prior research by the lab were not significant in the logistic regression analyses of this pilot study.

Discussion:

Based on multiple logistical regressions controlling for age and FDR, the plasma metabolomic profiles of 56 subjects with AMD and 19 control subjects older than 50 years differed significantly and consistently for 1 metabolite, alpha-tocopherol (*q*-value <0.05), which supports a similar metabolite finding in Dr. Husain's prior research on AMD metabolomic profiles. Prior research conducted by the lab found 20 metabolites that differed for macular degeneration patients compared to controls. These metabolites were mainly related to amino acids, lipids, and nucleic acids. Their methodology utilized two analytic methods to identify the metabolomic profile differences between AMD patients and controls: A) multivariable logistic regression analyses with AMD versus control as the outcome and B) permutation- based cumulative logistic regression models of both eyes with severity stage of disease as an outcome.[27] Between these two methods, alpha-tocopherol (AT) was the only additional metabolite found with the latter methodology. This initial pilot study, which similarly utilized both patient eyes in all stages of AMD, consistently demonstrated AT as a significant metabolite that differed between AMD patients and controls in their ability to dark adapt.

The significance of AT supports a potential AMD pathologic mechanism related to inflammation and oxidative stress. AT is the most abundant form of vitamin E in the body and can be found in seeds, nuts, leafy green vegetables, and vegetable oils.[28] Its role in the body is to prevent free radical cell damage to tissues and unsaturated lipids by acting as a scavenger of active oxygen radicals and by forming complexes with destabilizing molecules.[29] Prior studies have been conducted looking at the therapeutic relationship between vitamin E and inflammation. Vitamin E can potentially regulate inflammation by inhibiting NF-κB and JAK-STAT signaling pathways and by suppressing generation of prostaglandins and leukotrienes. Numerous animal studies have also shown protective benefits from vitamin E for pathologic conditions associated with inflammation and oxidative stress such as lung inflammation, colitis, allergic dermatitis, and pancreatitis.[28] Based on the results from this pilot study, it may be possible that the presence of alpha-tocopherol may be significantly higher in AMD patients than in

controls due to its role in regulating inflammation, another potential pathogenic mechanism of AMD. The presence of alpha-tocopherol in AMD patients during dark adaptation may also suggest its increased importance as an antioxidant given its multi-faceted role in reducing reactive oxygen species (ROS) and oxidative stress under multiple settings: A) in the normal retina, B) in AMD, and C) after bright light exposure. Normally, the retina is under increased risk of oxidative stress due to increased ROS from photosensitive molecules (rhodopsin and lipofuscin), higher oxygen metabolism, and retinal illumination.[30,31] On top of the regular oxidative stress placed on the retina, the retinas of AMD patients are at even higher risk for oxidative stress due to increased age-related accumulation of lipofuscin and increased lipid peroxidation of lipid/protein deposits, which may accumulate due to chronic local inflammation in AMD.[31] Photoreceptors responsible for the visual cycle also undergo intense oxidative metabolism in order to constantly turnover molecules. Furthermore, light, itself, can cause increased lipid peroxidation due to the DHA content of rods and rhodopsin activation during the phototransduction process has been associated with photoreceptor cell death secondary to oxidative stress. [31,32] It is not known why the other metabolites found to be significant in Dr. Husain's prior research were not found to be significant in relation to dark adaptation. One possible theory may be due to the increased oxidative stress placed on rods during the dark adaptation process. Our finding of AT for AMD patients during dark adaptation suggests its important antioxidant role in reducing the oxidative stress that may be going on secondary to AMD and bright light exposure.

This study has several limitations. First, our analyses did not account for severity stage of AMD as a possible confounder, making it difficult to determine if alpha-tocopherol was uniquely associated with the ability to dark adapt in AMD patients rather than to the severity stage of disease. Further analyses must be done to characterize whether severity stage of AMD is a possible confounder or an effect modifier. We also did not analyze the relationship between plasma metabolomic findings and dietary patterns and genetic risk profiles of patients and controls. Given that metabolites reflect the downstream nature of genetic transcription and its interaction with the environment, accounting for dietary and genetic risk profiles would further characterize the differences

of metabolic profiles for AMD and controls during dark adaption. Additionally, although multiple time points were utilized for dark adaption in order to increase the power of the study, the sample size of 56 AMD patients and 19 controls further limits presentation of more statistically significant results. Lastly, the study could be enhanced by finding more analytic methods and equations that better fit the model to the dark adaption curves of both eyes, such as through the use of non-linear mixed effects regression.

Despite these limitations, this study was an interesting pilot project to assess the plasma metabolomic profiles of AMD patients in all stages of disease and their ability to dark adapt. The work provides possible evidence that patients with AMD present an altered plasma metabolomic profile as compared to controls in relation to dark adaptation, possibly due to increased oxidative stress. Our study further supports oxidative stress pathways as a potential mechanism for AMD. Further advanced analyses with parameters accounting for additional confounders may help elucidate definitive causes of dark adaption in AMD patients, uncover pathogenic drivers of AMD, and identify potential biomarkers correlated to the decline of dark adaptation to measure for AMD progression. If these are identified in the future, they can contribute to the early diagnosis, screening, and prognosis of this blinding condition.

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Tables and Figures

Table 1. Characterization of Patients Included in the Study			
	AMD	Control	Total
Demographics and clinical characteristics			
Number, n (%) of eyes	100 (75)	33 (25)	133 (100)
Included eye, n (%)			
OD	48 (48)	17 (51.5)	65 (48.9)
OS	52 (52)	16 (48.5)	68 (51.2)
Number (%) of participants	56 (74.7)	19 (25.3)	75 (100)
Age, mean ± SD	69.2 ± 0.9	66.1 ± 1.8	68.4 ± 0.8
Gender, n (%)			
Male	21 (37.5)	11 (57.9)	32 (42.7)
Female	35 (62.5)	8 (42.1)	43 (57.3)
Race/ethnicity, n (%)			
White	50 (90.9)	16 (88.9)	66 (90.4)
Hispanic	4 (7.3)	1 (5.6)	5 (6.9)
Black	0 (0)	1 (5.6)	1 (1.4)
Asian	1 (1.8)	0 (0)	1 (1.4)
Eye examination and past history			
BCVA (logMAR), mean ± SD (range)	0.1 ± 0.02 (0.07, 0.13)	0.05 ± 0.02 (0.01,0.08)	0.09 ± 0.01 (0.06, 0.11)
Spherical equivalent (diopters), mean ± SD	0.5 ± 0.15	-0.5 ± 0.45	0.23 ± 0.16
IOP (mm Hg), mean ± SD	15.2 ± 0.29	14.0 ± 0.47	14.9 ± 0.25
BMI, mean ± SD	27.8 ± 0.46	26.5 ± 0.65	27.5 ± 0.38
Smoking, n (%)			
Non-smoker	29 (51.8)	8 (42.1)	37 (49.3)
Ex-smoker	26 (46.4)	10 (52.6)	36 (48.0)
Smoker	1 (1.8)	1 (5.3)	2 (2.7)
AMD= age-related macular degeneration; BCVA= best-corrected visual acuity; IOP= intraocular pressure; logMAR= logarithm of the minimal angle of resolution; OD= right eye; OS= left eye			

Table 2. Age-Related Macular Degeneration Stage and Rod Intercept Time for Study Eyes

	Number (%) of Eyes	RIT Mean (IQR)
AMD stage, n (%)		
Control	33 (24.8)	5.1 (4.24- 6.06)
Early AMD	25 (18.8)	6.2 (4.62-7.83)
Intermediate AMD	57 (42.9)	15.3 (13.86-16.70)
Late AMD	18 (13.5)	14.13 (10.91-17.35)
Total	133 (100)	10.91 (9.75-12.07)

AMD= age-related macular degeneration; IQR= interquartile range (in minutes); RIT= rod intercept time (in minutes).

Table 3. Significant Metabolites Associated with Dark Adaptation (RIT) in AMD and Control Patients using Generalized Linear Regression

Metabolite ID	Biochemical Name	Super Pathway	Beta	Standard Error	*P*-val_wald	*P*-value	*Q*-value
X1123	inosine	Nucleotide	3.031734	2.010054	0.1339082	0.1287683	0.0148401
X1561	alpha-tocopherol	Cofactors and Vitamins	3.550543	4.387713	0.4198779	4.14E-01	9.50E-04
X1572	glycerate	Carbohydrate	-11.01678	4.880202	0.0256483	0.0237328	0.0065025
X1587	N-acetylleucine	Amino Acid	1.0063	9.97169	0.9197729	0.9187	0.0383544
X20676	maleate	Lipid	-15.08431	7.418744	0.0440609	0.0412984	0.0473735
X20694	oxalate (ethanedioate)	Cofactors and Vitamins	-8.938509	5.241796	0.0905384	0.0862954	0.0189426
X27738	threonate	Cofactors and Vitamins	-3.529355	6.88118	0.6088919	0.6040942	0.0148401
X32497	10-undecenoate (11:1n1)	Lipid	2.158543	3.107845	0.488579	0.4827638	0.0383544
X44656	isovalerate (i5:0)	Amino Acid	-13.65759	9.46257	0.1513337	0.145922	0.0396434
X46548	3-methylglutarylcarnitine (2)	Amino Acid	11.61977	4.866291	0.0183881	0.0168791	0.048673
X5086	dimethylglycine	Amino Acid	-2.161853	6.158017	0.7261093	0.7225846	0.0353727
X514	cytidine	Nucleotide	-8.486473	6.781682	0.2130424	0.2069663	0.0092248
X52473	gamma-tocopherol/beta-tocopherol	Cofactors and Vitamins	-54.71585	29.42978	0.0652596	0.0617333	0.00254
X52616	1-palmitoyl-2-stearoyl-GPC (16:0/18:0)	Lipid	-23.98298	11.64022	0.0413594	0.0387084	0.0383544
X53010	lactosyl-N-palmitoyl-sphingosine (d18:1/16:0)	Lipid	-6.642492	4.47804	0.1404023	0.135156	0.0403523
X54923	beta-citrylglutamate	Amino Acid	11.96248	5.509823	0.0317382	0.0295178	0.0027455
X57370	lactosyl-N-nervonoyl-sphingosine (d18:1/24:1)*	Lipid	-8.465785	8.52823	0.3227111	0.3162608	0.0329451
X57450	stearoyl-arachidonoyl-glycerol (18:0/20:4) [1]*	Lipid	-9.714742	9.141651	0.2898927	0.2834693	0.0362522
X59	histidine	Amino Acid	4.847299	6.700087	0.4706922	4.65E-01	9.50E-04

Table 4. Significant Metabolites Associated with Dark Adaptation (Continuous Time) in AMD and Control Patients using Linear Mixed-Effects Regression Model Accounting for Random Eye and Patient Effects

Metabolite ID	Biochemical Name	Super Pathway	Beta	Standard Error	AIC	*P*- value_wald	*P*- value	*Q*-value
X1561	alpha-tocopherol	Cofactors and Vitamins	-2.321377	0.542228	1121.322	0.0000194	0.0000368	0.0105789
X1572	glycerate	Carbohydrate	-2.827822	0.7458895	1124.701	0.0001542	0.00022	0.0419541

Table 5. Significant Metabolites Associated with Dark Adaptation (Continuous Time) in AMD and Control Patients using Linear Mixed-Effects Model Model Accounting for Autoregression

Metabolite ID	Biochemical Name	Super Pathway	Beta	Standard Error	Likelihood Ratio Test	AIC	*P*- value	*Q*-value
X1561	alpha-tocopherol	Cofactors and Vitamins	-2.342708	0.5632505	0.9958322	35.53279	0.0000871	0.0180837