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Daytime Lipid Metabolism Modulated by *CLOCK* Gene Is Linked to Retinal Ganglion Cells Damage in Glaucoma

Denis Gubin [1,2,3,*], Vladimir Neroev [4], Tatyana Malishevskaya [4], Sergey Kolomeichuk [5,6], Dietmar Weinert [7], Natalya Yuzhakova [5], Alsu Nelaeva [8], Yulia Filippova [9] and Germaine Cornelissen [10]

1 Laboratory for Chronobiology and Chronomedicine, Research Institute of Biomedicine and Biomedical Technologies, Medical University, 625023 Tyumen, Russia
2 Department of Biology, Medical University, 625023 Tyumen, Russia
3 Tyumen Cardiology Research Center, Tomsk National Research Medical Center, Russian Academy of Science, 634009 Tomsk, Russia
4 Helmholtz Research Institute of Eye Diseases, 105062 Moscow, Russia; sekr@igb.ru (V.N.); malishevskoff@yandex.ru (T.M.)
5 Laboratory for Genomics, Metabolomics and Proteomics, Research Institute of Biomedicine and Biomedical Technologies, Medical University, 625023 Tyumen, Russia; sergey_kolomeychuk@rambler.ru (S.K.); yuzhakova@tyumsmu.ru (N.Y.)
6 Laboratory of Genetics, Institute of Biology of the Karelian Science Center of the Russian Academy of Sciences, 185910 Petrozavodsk, Russia
7 Department of Zoology, Institute of Biology/Zoology, Martin Luther University, 06108 Halle, Germany; dietmar.weinert@zoologie.uni-halle.de
8 Department of Endocrinology, Medical University, 625023 Tyumen, Russia; nelaeva@inbox.ru
9 State Autonomous Health Care Institution Tyumen Regional Ophthalmological Dispensary, 625048 Tyumen, Russia; 74174186@mail.ru
10 Halberg Chronobiology Center, University of Minnesota, Minneapolis, MN 55455, USA; corne001@umn.edu
* Correspondence: dgubin@mail.ru

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Featured Application: Compromised light perception, as with retinal ganglion cell loss, is associated with serum lipids being unevenly altered in the morning vs. the evening, depending on *CLOCK* gene polymorphism. One should consider this in the diagnosis of retinal and metabolic conditions.

Abstract: Lipid metabolism is intimately linked to circadian mechanisms and light signaling. Deteriorated photic transduction because of retinal ganglion cell (RGC) loss occurring with glaucoma progression reduces perceived light amplitude, causing circadian disruption. To investigate associations with RGCs, total cholesterol (TC), its low-density (LDL-C) and high-density (HDL-C) fractions, and triglycerides (TG) were measured, under a controlled meal regimen, during daytime hours in 114 patients diagnosed with primary open-angle glaucoma (POAG). RGC damage was assessed by high-definition optical coherence tomography (HD-OCT). Analysis of eight clock, clock-related, and melatonin receptor gene polymorphisms was performed on 19 patients. RGC loss was associated with changes in lipid metabolism in a time-dependent manner. Morning (08:00) values of HDL-C ($r = 0.613$, $p < 0.0001$) and TG ($r = 0.568$, $p < 0.0001$) correlated positively with RGC global loss, while LDL-C at 08:00 had a weak correlation ($r = 0.235$; $p = 0.012$) but showed a strong correlation in the evening (20:00) ($r = 0.533$, $p < 0.0001$). The morning–evening gradients (MEGs, changes at 20:00 versus 08:00) in TC and LDL-C changed sign from a negative to a positive association in patients exceeding the 15% two-eye mean GLV threshold. MEG (LDL-C higher in the evening than in the morning) was positive only in POAG patients with the CLOCK_3111 TT genotype.

Keywords: glaucoma; retinal ganglion cells; metabolism; lipids; cholesterol; triglycerides; circadian disruption; CLOCK_3111; SNP rs1801260

1. Introduction

Lipid metabolism is entwined with the circadian machinery, and its alterations are closely associated with circadian disruption [1–15]. One factor that causes deterioration in circadian rhythms is glaucomatous damage to retinal ganglion cells (RGCs), which compromises light perception and signal transduction to the master clock (suprachiasmatic nuclei in the brain). RGC damage was found to be associated with disrupted local [16,17] and systemic [18–21] circadian rhythms, as well as sleep [22,23]. Circadian rhythms of lipid metabolism are modulated by light and food [5–15]. Furthermore, light and the extent of the day–night intensity differences modulate chronotype, sleep parameters, and circadian alignment [24–28]. This also occurs in glaucoma [29]. Seasonal differences in serum lipids [30,31] can in part be accounted for by differences in ambient light conditions [32,33]. There are substantial differences in circadian and seasonal phases in the rhythms of different serum lipids [31]. Recently, phase response curves (PRCs) of circadian lipid rhythms to light and food that appeared to differ between lipid fractions (e.g., LDL-C failed to demonstrate a PRC) were characterized [15].

On the other hand, dyslipidemia is associated with an increased risk of glaucoma and elevated intraocular pressure (IOP) [34]. Specific associations of glaucomatous damage and RGC loss with changes in total cholesterol, its low-density (LDL-C) and high-density (HDL-C) fractions, and triglycerides, (TG) were previously reported [34–37]. Taken together, changes in lipid metabolism can be both a factor predisposing to RGC damage in glaucoma and a consequence of RGC loss, which reduces the amplitude of perceived daylight photic information and compromises circadian rhythms.

To the best of our knowledge, data on lipid metabolism collected at different day times in relation to RGCs loss in glaucoma are currently lacking. Therefore, this study investigated how changes in serum lipids, assessed at different times of the day, depend on RGC loss that occurs with glaucoma. The role of clock genes, melatonin receptor gene polymorphisms, and salivary melatonin profiles was assessed in a subgroup of patients.

2. Results

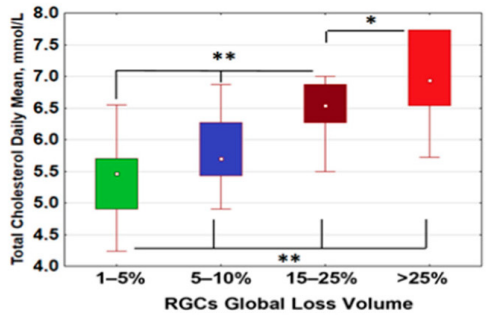
2.1. Lipid Metabolism Was Associated with Retinal Ganglion Cells Loss

Daytime mean (DM) of TC gradually increased with RGCs loss, being higher with each 5% GLV% increase, Figure 1. Higher DM of TG and LDL-C and lower DM of HDL-C were linked to GLV% values above 15%. DM of LDL-C in the GLV 15–25% subgroup was higher than in the GLV 1–5% subgroup.

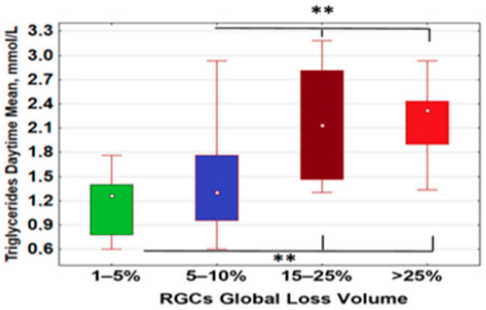
2.2. Changes of Lipid Metabolism during the Day Depend on RGC Loss. Evening–Morning Gradient of Cholesterol and Low-Density Lipids Was Associated with Retinal Ganglion Cells Loss

RGC loss above 15% was associated with higher TG and lower HDL-C, with no change in the daytime pattern of these lipids. Higher TC and LDL-C were associated with glaucoma progression, and with changes in their daytime pattern, Figure 2, as indicated from ANOVA with time and degree of RGC damage as cofactors.

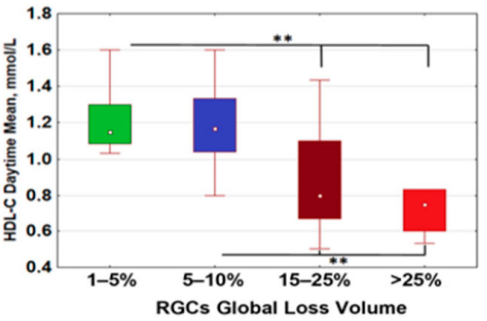
Most prominent were changes between morning (08:00) and evening (20:00) values of serum lipids. We defined such changes as the morning–evening gradient (MEG), which was investigated in four subgroups at different stages of GLV. An evening increase instead of a decrease in TC and LDL-C was evident in patients with RGC loss above 15%, and even more so in patients with RGC loss above 25%, Figure 2. Linear regression analysis confirmed the stronger correlation of LDL-C with RGC loss and GLV%, in the evening as compared to morning, Figure 3 (Dunn and Clark's $z = -5.069$, $p < 0.0001$). MEGs notably changed sign from a decrease to an increase at the RGC GLV 15% threshold. Only the LDL-C MEGs increased further for RGC damage above 25%, Figure 4.



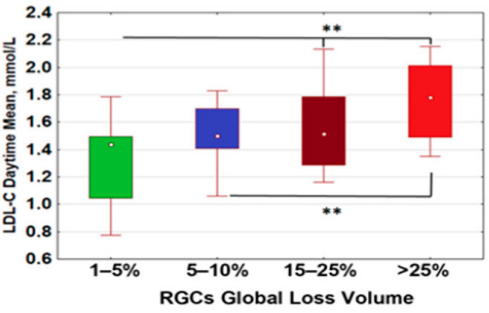
P-values from Tukey HSD test: **TC daytime mean**				
GLV %, mean Mean TC:	**1–5% 5.326**	**5–10% 5.832**	**15–25% 6.517**	**>25% 6.964**
1–5%		0.006	0.001	0.001
5–10%	0.006		0.001	0.001
15–25%	0.001	0.001		0.040
>25%	0.001	0.001	0.040	



P-values from Tukey HSD test: **TG daytime mean**				
GLV %, mean Mean TG:	**1–5% 1.176**	**5–10% 1.388**	**15–25% 2.211**	**>25% 2.216**
1–5%		0.535	0.001	0.001
5–10%	0.535		0.001	0.001
15–25%	0.001	0.001		0.999
>25%	0.001	0.001	0.999	



P-values from Tukey HSD test: **HDL-C daytime mean**				
GLV %, mean Mean HDL-C:	**1–5% 1.248**	**5–10% 1.187**	**15–25% 0.873**	**>25% 0.860**
1–5%		0.816	0.001	0.003
5–10%	0.816		0.001	0.002
15–25%	0.001	0.001		0.998
>25%	0.003	0.002	0.998	



P-values from Tukey HSD test: **LDL-C daytime mean**				
GLV %, mean Mean LDL-C:	**1–5% 1.319**	**5–10% 1.481**	**15–25% 1.561**	**>25% 1.767**
1–5%		0.174	0.021	0.001
5–10%	0.174		0.548	0.004
15–25%	0.021	0.548		0.839
>25%	0.001	0.004	0.839	

Figure 1. Daytime means of serum lipids in glaucoma patients with different degrees of retinal ganglion cell (RGC) loss, assessed as two-eye mean global loss volume (GLV%), gauged by high-definition optical coherence tomography. n = 114; RGC GLV < 5%, n = 16; GLV = 5–10%, n = 49, GLV = 15–25%, n = 35; n > 25%, n = 14). Effects for daytime means: total cholesterol, TC: KW-H $_{(3;114)}$ = 54.923; $p < 0.0001$; triglycerides, TG: KW-H $_{(3;114)}$ = 47.120; $p < 0.0001$; HDL-C: KW-H $_{(3;114)}$ = 34.988; $p < 0.0001$; LDL-C: KW-H $_{(3;114)}$ = 12.762; $p = 0.005$. GLV%: two-eye mean RGC global loss volume. *p*-values for statistically significant difference between groups after Tukey's honestly significant difference (HSD) test post-hoc are shown in red. **: $p < 0.01$; * $p < 0.05$.

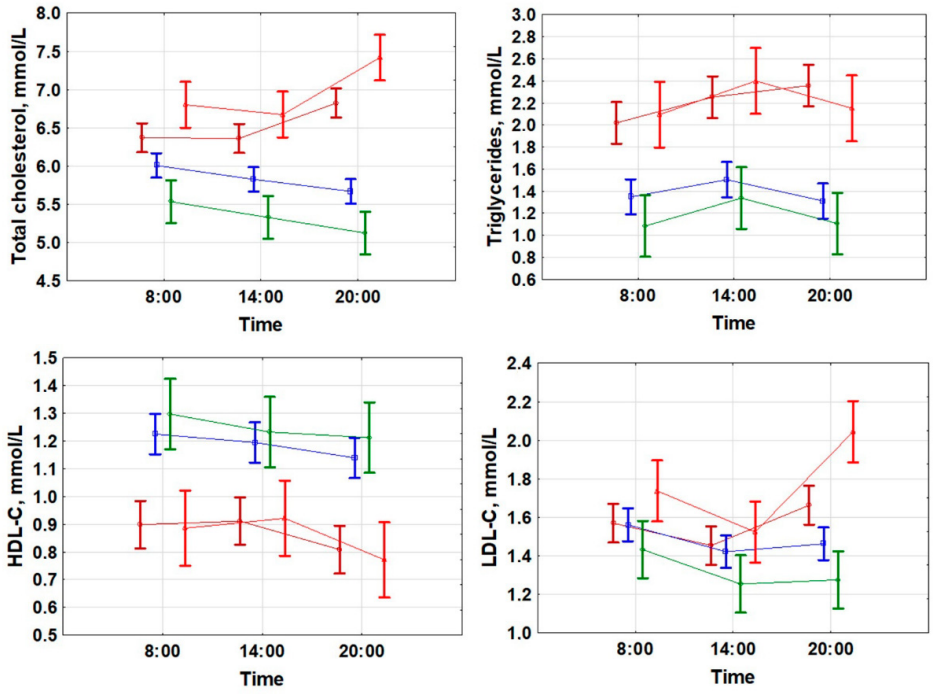


Figure 2. Daytime patterns of lipid metabolism in glaucoma patients with different degrees of retinal ganglion cell (RGC) loss, assessed as two-eye mean global loss volume (GLV%), gauged by high-definition optical coherence tomography. n = 114; RGC GLV < 5%, n = 16; GLV = 5–10%, n = 49, GLV = 15–25%, n = 35; n > 25%, n = 14). Effects for time–group interaction: total cholesterol: $F_{(6, 330)} = 6.267$, $p < 0.0001$; triglycerides: $F_{(6, 330)} = 1.006$, $p = 0.421$; high-density-lipid cholesterol, HDL-C: $F_{(6, 330)} = 0.247$, $p = 0.960$; low-density lipid cholesterol, LDL-C: $F_{(6, 330)} = 3.351$, $p = 0.003$. **GLV < 5%; GLV 5–10%; GLV 15–25%; GLV > 25%.**

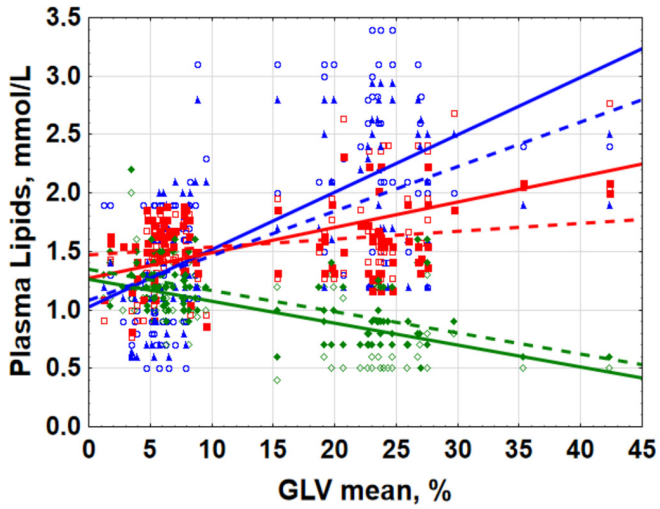
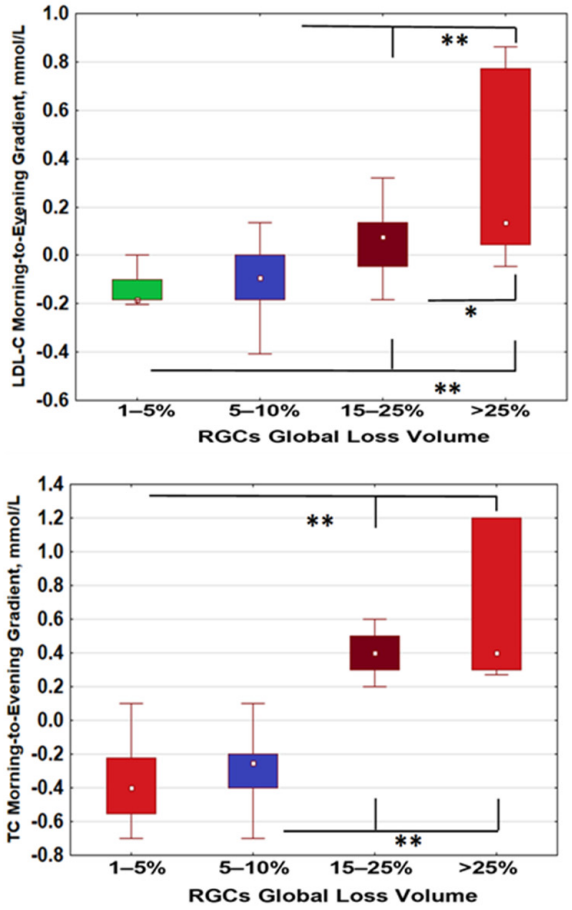


Figure 3. Changes in serum lipids associate with retinal ganglion cell (RGC) loss (two-eye mean global loss volume, %) in glaucoma. Evening (20:00) low-density lipid cholesterol (LDL-C) correlated more strongly with GLV% than morning (08:00) LDL-C; Dunn and Clark's $z = -5.069$, $p < 0.0001$. strongly with GLV% than morning (08:00) LDL-C; Dunn and Clark's $z = -5.069$, $p < 0.0001$. n = 114. TG at 08:00 (r = 0.568; $p < 0.001$); TG at 20:00 (r = 0.608; $p < 0.001$). HDLP at 08:00 (r = −0.613; $p < 0.001$); HDL-C at 20:00 (r = −0.585; $p < 0.001$). LDLP at 08:00 (r = 0.235; p = 0.012); LDL-C at 20:00 (r = 0.533; $p < 0.001$).



P-values from Tukey HSD test: **TC MEG**				
GLV %, mean TC MEG:	**1–5% −0.409**	**5–10% −0.340**	**15–25% 0.455**	**>25% 0.619**
1–5%		0.9314	0.0001	0.0001
5–10%	0.9314		0.0001	0.0001
15–25%	0.0001	0.0001		0.5692
>25%	0.0001	0.0001	0.5692	

P-values from Tukey HSD test: **LDL-C MEG**				
GLV %, mean LDL-C MEG:	**1–5% −0.157**	**5–10% −0.098**	**15–25% 0.093**	**>25% 0.307**
1–5%		0.7899	0.0016	0.0001
5–10%	0.7900		0.0010	0.0001
15–25%	0.0016	0.0010		0.0142
>25%	0.0001	0.0001	0.0142	

Figure 4. Morning–evening gradients (MEGs) of total cholesterol (TC, left) and low-density lipid cholesterol (LDL-C, right) depended on retinal ganglion cells global volume loss (GLV%). n = 114; RGC GLV < 5%, n = 16; GLV = 5–10%, n = 49, GLV = 15–25%, n = 35; n > 25%, n = 14). Note that MEG was *decreasing* in individuals with GLV% lower than 10% and changed sign to *increasing* in individuals with RGC GLV of 15% or more; LDL-C MEGs increased further in patients with GLV% above 25%. TC: KW-H $_{(3;114)}$ = 71.776; $p < 0.0001$; LDL-C: KW-H $_{(3;114)}$ = 41.933; $p < 0.0001$. GLV%: two-eye mean retinal ganglion cells' global loss volume. *p*-values are in red for the differences between groups after adjustment for Tukey's Honestly Significant Difference (HSD) post-hoc test. **: $p < 0.01$; * $p < 0.05$. □ Median; ▭ 25–75%; ⊥ Non-Outlier Range.

Since serum lipids also change as a function of age, we used age as a co-factor in the multiple regression assessing the effect of lipids on RGC GLV, Table 1. We found that GLV was more strongly associated than age with TG, HDL-C, or TC, both in the morning and in the evening. LDL-C surpassed age, however, only in the case of evening values and MEGs. Strong positive associations between MEGs and RGC GLV% were evident for all studied serum lipids (TG, b = 0.417 ± 0.089; TC, b = 0.720 ± 0.066; LDL-C, b = 0.557 ± 0.078), except for HDL-C (not significant). Of note, the relationship of TG DM with age (negative correlation, b = −0.320 ± 0.089) was opposite to that with GLV% (positive correlation, b = 0.417 ± 0.089), Table 1.

Table 1. Linear regression and multiple stepwise regression model of daytime lipids as a function of retinal ganglion cell damage and age.

Dependent Variable	GLV, r Value	Age, r Value	All-Effects Multiple Regression Model, b-Value(s)	Backward Stepwise Regression Model, b-Value(s)
Morning (08:00)				
Cholesterol	**0.526**	**0.365**	**GLV, b = 0.458** **Age, b = 0.231**	**GLV, b = 0.526**
TG	**0.568**	0.120	**GLV, b = 0.583** Age, b = −0.050	**GLV, b = 0.568**
HDL-C	**−0.613**	−0.083	**GLV, b = −0.640** Age, b = 0.104	**GLV, b = −0.610**
LDL-C	**0.235**	**0.294**	**Age, b = 0.247** GLV, b = 0.163	**Age, b = 0.294**
Evening (20:00)				
Cholesterol	**0.774**	**0.350**	**GLV, b = 0.734** **Age, b = 0.137**	**GLV, b = 0.774**
TG	**0.608**	0.017	**GLV, b = 0.659** **Age, b = −0.170**	**GLV, b = 0.608**
HDL-C	**−0.585**	−0.070	**GLV, b = −0.620** Age, b = 0.109	**GLV, b = 0.580**
LDL-C	**0.533**	**0.403**	**GLV, b = 0.454** **Age, b = 0.271**	**GLV, b = 0.454** **Age, b = 0.271**
Morning–Evening Gradients (MEGs)				
Cholesterol	**0.721**	**0.185**	**GLV, b = 0.728** Age, b = −0.030	**GLV, b = 0.720**
TG	**0.323**	**−0.201**	**GLV, b = 0.417** **Age, b = −0.320**	**GLV, b = 0.417** **Age, b = −0.320**
HDL-C	−0.049	0.013	GLV, b = −0.060 Age, b = 0.029	—
LDL-C	**0.558**	**0.297**	**GLV, b = 0.514** Age, b = 0.147	**GLV, b = 0.557**

GLV—retinal ganglion cells' global loss volume, %; TG—triglycerides; HDL-C—high-density-lipid cholesterol; LDL-C—low-density lipid cholesterol. n = 114. Significant correlations are bold. —: no significant associations for HDL-C with MEGs.

2.3. Morning–Evening Gradient of Low-Density Lipids Was Linked to Retinal Ganglion Cell Loss and CLOCK Gene Polymorphism

A subgroup of 19 patients was used to study associations between lipid patterns and MEGs, and between lipid patterns, MEGs and clock, clock-controlled, and melatonin-receptor genes. Fifteen of these patients were also used to study salivary melatonin profiles. No significant associations were found between lipid patterns, MEGs, and parameters of salivary melatonin (mean, circadian amplitude, circadian phase, or peak time). Among gene polymorphisms, a significant association was found between CLOCK_3111 rs1801260 and LDL-C MEG, Table 2. This single nucleotide polymorphism, SNP, was previously linked to the sleep–wake cycle, body weight, and lipid metabolism. Some studies considered minor C allele as protective, linked to lower cholesterol in Russian women [38]. It should be considered, however, that only one patient was homozygous for the minor C allele of CLOCK_3111 (rs1801260), genotype CC. Twelve patients had the TC genotype and another six were homozygous in TT. The evening rise in LDL-C was linked to the CLOCK_3111 (rs1801260) TT genotype; KW-H (1;18) = 5.500; $p = 0.019$, Figure 5. Only carriers of the TT genotype had an increase in LDL-C MEG, with higher values at 20:00 than at 08:00. Such an

increase was not evident in carriers of the TC genotype who tended to have a lower LDL-C MEG. There was no interaction between CLOCK_3111 TC polymorphism and glaucoma progression; F (1, 14) = 0.002, $p = 0.967$, Supplementary Materials Figure S1, indicating that both factors, TT genotype and RGC loss, are independently associated, or even potentiate the increase in LDL-C MEG.

Table 2. Morning–evening gradients (MEGs) of low-density lipid cholesterol (LDL-C) depended on studied polymorphic variants of the biological clock, melatonin receptors, and G-protein genes in primary-open glaucoma patients (n = 19). Significant correlations are bold.

Gene Polymorphisms	*p*-Value for Effect Significance; ANOVA for LDL-C MEG
***CLOCK* rs1801260 3111T/C**	**0.023**
PER2 rs6431590	0.507
PER3 VNTR	0.644
CRY1 rs12820777	0.169
MTNR1A rs34532313	0.656
MTNR1B rs10830963	0.338
GNB rs5443	0.814

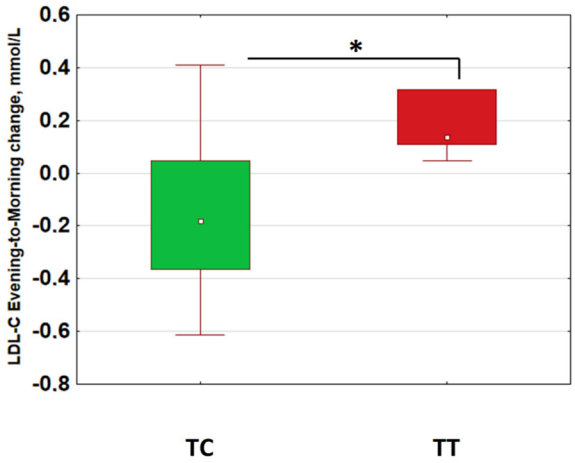


Figure 5. Evening rise in low-density lipid cholesterol, LDL-C was linked to CLOCK_3111 (rs1801260) TT genotype. Kruskal–Wallis, KW-H $_{(1;18)} = 5.500$; $p = 0.019$. n = 18.; TC = 12; TT = 6 *: $p < 0.05$.

3. Discussion

Lipid metabolism and the circadian machinery are closely interrelated. Disruption of circadian rhythms in glaucoma is caused by progressive damage to RGCs [18–20,39–41], particularly to intrinsically photosensitive retinal ganglion cells, ipRGCs [22,42]. Altered lipids were repeatedly shown to be associated with glaucoma [36,37] and commonly considered as one of the putative causative co-factors. However, an alternate concept can be proposed, linking altered lipid metabolism with compromised light perception and concomitant circadian misalignment. Indeed, several recent works demonstrated that circadian disruption due to compromised light perception, or diminished amplitude of circadian light signaling may affect metabolism [1,15,43,44]. RGC loss in glaucoma diminishes the amount of daylight perceived and transferred to the relevant brain regions, including the SCN. Our results reported herein suggest that changes in serum lipids could be integrated into a vicious circle caused by circadian disruptions due to glaucomatous damage to RGCs [21]. One may assume a causal relationship between progressively impaired daylight photic signaling and glaucoma progression and specific alterations of lipid metabolism, since changes in light perception may influence circadian lipid metabolism that may involve,

or not involve changes in daily food consumption. Dimmer diurnal light, similarly to light at night, reduces the amplitude of ambient light, the main circadian synchronizer, thereby weakening circadian entrainment and altering lipid metabolism. Indeed, circadian disruption is caused by clock gene mutations [1,45,46], or changes in ambient light conditions [1,43,44], which have numerous metabolic consequences. These studies suggest that diminished amplitude of light signaling between day and night, including dim light at night, may cause lipid metabolism alteration resembling what we found with progressive loss of RGCs in glaucoma. Contrary to light-at-night, when the circadian amplitude is diminished due to excess light when light should be avoided, in glaucoma patients, the circadian amplitude is diminished due to light deficit during the daytime. Since RGCs are necessary to convey light signals to the central circadian oscillator, their progressive loss reduces the effectiveness of the synchronizing role of circadian rhythms by the environmental light–dark cycle. In this study, changes in lipid metabolism were not only associated with RGC loss, but they also depended on their daytime pattern. Morning values of HDL-C and TG gradually increased with RGC loss. On the contrary, morning LDL-C values correlated only weakly with RGC loss. A stronger correlation was found in the evening, which was associated with a larger morning–evening gradients (MEGs). Moreover, MEGs were influenced by both RGC loss and *CLOCK* gene polymorphism.

Current knowledge of circadian rhythmicity of serum lipids is scarce and varies between populations and depending on study conditions. Twenty-four-hour profiles of serum lipids vary among individuals. In real-life conditions, 24 h rhythms in TC and HDL-C have a phase in the early evening (peaking around 16:00–17:00 h). A phase advance of approximately 2 h (to around 14:00–15:00) was reported after the age of 60 in healthy adults in India [47], with similar results in Spain [48], later confirmed with a higher-density sampling in the US [49]. A study in Japan found that both LDL-C and HDL-C decrease during the day, both fractions being significantly lower around midnight than after breakfast [50]. Another study conducted in the Republic of North Macedonia reported no 24 h rhythm in HDL-C [51]. The partly contradictory results may be explained by high intra-individual variability in 24 h and 12 h phasing [52] and high inter-individual [49] variability, and by differences in phenotypes of circadian patterns of lipid metabolism. Such differences may depend on the circadian phase of the intrinsic clock [6,15], which governs sleep and food consumption, and therefore must either directly or indirectly affect circadian profiles of lipid metabolism. Of note, postprandial responses of serum lipids to the same meal depend on time, with the effect in the morning higher than in the afternoon [14]. Furthermore, altered lipid metabolism, higher cholesterol, LDL-C [52], and atherogenic ratio (LDL-C/HDL-C) were found in evening chronotypes [53,54]. Recently, a constant routine study was conducted [15] that supported early evening mean phases for circadian rhythms of TC (18:32), LDL-C (17:00), and HDL-C (19:17). This study reported that the nocturnal phase for TG (03:22) was very close to that of melatonin (03:41) [15]. The authors also showed phase response curves (PRCs) for lipids (TC, HDL-C, and TG) in response to light and food, with greater shifts for lipids compared to melatonin, approximately in antiphase to melatonin [15]. These findings provide strong evidence that ambient light drives circadian patterns and phases of serum lipids. Interestingly, this study also showed that, unlike other lipids, LDL-C failed to demonstrate a PRC in response to light, suggesting that the mechanisms driving LDL-C are somewhat distinct. This result is intriguing since in our study, morning LDL-C also failed to demonstrate a robust correlation with RGC loss, whereas other lipids did. Moreover, only LDL-C showed MEGs that were attributed to a certain *CLOCK* gene SNP.

Another source of evidence that blood lipids depend on ambient light conditions stems from studies demonstrating differences in serum lipids in photoperiodic conditions of different seasons and latitudes. For example, LDL-C, HDL-C, and its turnover by lipase enzymes have seasonal differences, depending on photoperiod or light exposure [30,55]; reviewed in [31]. There are many discrepancies, especially in TC and LDL-C concerning the season when their peak phase occurs. Gender and age also matter. However, most

studies agree that the lowest values are usually seen during the summer, the season also corresponding to the highest light exposure. One study found that summer, the season with an increased light phase, is linked to lower LDL-C and higher HDL-C [55], favoring the hypothesis that the amount of light received per 24 h via the retina could be associated with bidirectional changes in cholesterol fractions. As the lowest value for LDL-C occurred in summer while that for HDL-CL occurred in winter, such reciprocal seasonal differences between LDL-C and HDL-C performed in a location with relatively narrow seasonal differences in ambient temperature (Spain) suggest that, yet again, such differences can be linked to a near 6 h difference in sunset between summer and winter.

The association of MEG in LDL-C with the *CLOCK* gene reported herein further strengthens the evidence that lipid metabolism is clock- and light-controlled. Numerous previous studies showed that the *CLOCK* gene is involved in lipid metabolism [56], and that its CLOCK_3111 TC (rs1801260) polymorphism matters particularly [38,57–63]. Since our study showed that *CLOCK* rs1801260 polymorphism accounts mainly for the differences in MEG, it may help to explain why no differences in serum lipids are found in studies relying only on morning measurements [61].

Remarkably, as far as morning values are concerned, our study reaches similar conclusions as those of a recent metanalysis, which reported considerably higher mean TC and lower mean HDL-C values in patients diagnosed with glaucoma than in patients without glaucoma, but no statistically significant difference in mean LDL-C values [37]. Common clinical practice consists of assessing lipids in the morning. Unlike other lipids, however, LDL-C is more predictive of impairment in the evening than in the morning, as shown herein. Not only in glaucoma, but also in other common pathologies affecting retinal light perception, such as in diabetic retinopathy, a lower HDL-C/LDL-C ratio was found to correlate with disease severity, assuming that light perception can be involved [63]. Furthermore, in a transgenic mouse model of neurodegenerative pathology (Alzheimer's disease), the diminished amplitude of circadian light signaling was associated with a higher amyloid load, whilst strong robust signaling correlated with a reduced amyloid load [64]. These results suggest that altered light perception and circadian disruption can facilitate a vicious cycle in the progression of neurodegenerative pathology that may include glaucoma [21]. Note that *CLOCK* gene rs1801260 polymorphism, yet again, can be involved [65].

Alterations to photic signaling and transduction cause disruption of circadian rhythms, the severity of which depends on impairment of ipRGCs, and melatonin production can also be affected by its receptor gene polymorphisms [19,21]. The fact that when visual function is lost, photic signaling is still transferred to the central clock prompted the discovery of ipRGCs [66–68]. Studies in totally blind people revealed that circadian rhythms of melatonin and cortisol were not equally compromised when light perception was not possible. Lockley and al. [69] described that 23% of individuals with no light perception remained entrained, and even more (37%) in another study [70], while the majority was non-entrained or had abnormal phase positions [69,70]. Individual differences may be either due to genetic polymorphisms in clock, clock-controlled, or melatonin receptors' genes, or to differences in non-photic cues that likely include food consumption. Since loss of light perception affects pineal and pituitary functions, down-streaming hormones are also compromised [71,72], leading to the occurrence of complex metabolic consequences. No statistically significant associations between lipids and parameters of salivary melatonin were found in our study. This result is likely due to the modest number of patients involved and the lack of lipid determinations during the nocturnal phase of the 24 h cycle. This study did not specifically address damage to ipRGCs, which is its limitation. Our findings of pronounced alterations that occur simultaneously in lipid metabolism, circadian rhythms, and sleep, specifically in patients with advanced glaucoma, however, are in good agreement with findings of Obara et al. [42] showing that specific damage to ipRGCs appears in advanced but not in mild stages of glaucoma.

This study was primarily observational in design. Therefore, patients were free to follow their habitual sleep–wake schedules, a design feature that could affect results.

However, the meal regimen was standardized in this study, suggesting that MEG differences are not derived from individual differences in diet or meal timing. Implementing circadian research for applied clinical purposes is important for translational circadian medicine [73,74]. This study is just one of this kind. Since light and feeding contribute to circadian alignment, further in-depth studies are needed to assess their roles. Light, being a principal synchronizer, can also modulate mealtimes. Circadian misalignment between feeding and clock affects anabolic–catabolic balance, including insulin sensitivity, and inflammation [75]. Further studies should also include feeding- and light-controlled protocols to obtain a more complete picture of interrelations within the vicious cycle of compromised light perception and altered metabolism in glaucoma and other diseases in which light perception is altered. It is known that circadian clocks are prone to be delayed due to reduced light signaling [25] that indeed occurs with RGC loss in glaucoma [19–21]. The question of whether changes in the circadian machinery of lipid metabolism can be fostered either directly by light signaling, or indirectly by means of changes in habitual diet, is thus pertinent. Both scenarios can be linked to the *CLOCK* gene. Whether accumulated daylight debt associates with impaired lipid metabolism in glaucoma is another question awaiting investigation.

4. Materials and Methods

This cross-sectional study adhered to the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board at the Tyumen Scientific Center of the Siberian branch of the Russian Academy of Sciences (Protocol No. 5, 15 May 2013). The work was included in the research plan of the Federal State Budgetary Institution of Science at the Tyumen Scientific Center of the Siberian branch of the Russian Academy of Sciences (registration number AAAA-A17-117120500038-2). Written informed consent was obtained from all participants according to the order of the Ministry of Health of the Russian Federation, No. 266 (19 March 2003). All patients were examined and diagnosed under the supervision of the State Autonomous Health Institution of the Tyumen Region "Regional Ophthalmological Dispensary" during 2013–2016. The current findings are based on data from a previously published cross-sectional study [17,19,20].

4.1. POAG Diagnosis and Progression Criteria

The criteria for selecting patients with POAG were visual acuity 0.5–1.0 (without correction or with correction requiring no more than ±3.0 diopters, and no more than 1 diopter for astigmatism), a transparent lens, and no pathology of the macular region of the retina. The dynamics of visual functions were assumed to have stabilized in patients with a change in mean deviation (mD) and visual field sensitivity deviation from normal values of peers with unaltered vision by no more than 0.5 decibels (dB) per year, and a decrease in GLV of no more than 2% per year. These patients were assigned to the stable group, S-POAG. In other cases, the process was considered progressive, and patients were assigned to the advanced group, A-POAG. Data from the worst eye were considered for group assignment. Patients with S-POAG and A-POAG were matched by gender, age, and treatment. Exclusion criteria were described previously [17,19,20].

4.2. RGC Damage Assessment

Standard automated perimetry (SAP) was performed to assess visual field (VF) with the Humphrey Field Analyzer (Carl Zeiss, Jena, Germany), using the 30-2 SITA-Standard strategy. The following parameters were obtained: total photosensitivity of the central VF, mean deviation (mD), and pattern standard deviation (PSD). Damage to the retinal ganglion cell complex (RGCC) was measured by means of high-definition optical coherence tomography (HD-OCT) (RTVue-100, Optovue, 2800 Bayview Dr, Fremont, CA, USA). The average amount of GCC loss over the entire GCC map (global loss volume, GLV, %) and the average amount of localized thinning over the entire GCC map (focal loss volume, FLV, %) were estimated. Optic nerve head (ONH) and retinal nerve fiber layer (RNFL) scanning

protocols 3.45 were used, GCC for the RTVue-100 tomograph. Depending on two-eye mean RGC GLV%, four groups were formed: 1. GLV = 1–5%; 2: GLV = 5–10%, GLV = 15–25%; and GLV > 25%. There were no patients with two-eye mean GLV% between 10 and 15%.

4.3. Plasma Lipid Assessment

Blood samples were collected while patients were admitted at a daytime patient facility. Samples of venous blood were collected three times a day, at 08:00, 14:00, and 20:00. Total cholesterol (TC), high-density lipid cholesterol (HDL-C), and triglycerides (TG) were assessed photometrically by Cormay kit (Łomianki, Poland) on a semiautomatic analyzer (Cormay, Łomianki, Poland). Low-density lipid cholesterol (LDL-C) was estimated by Friedewald's formula [76] as TG were below 4.5 mmol/L in all samples. As one patient failed to complete sample collection, data from 114 patients were included for analysis. Daytime mean (DM) corresponded to the individual average of the three consecutive serum lipid measurements (08:00–14:00–20:00). A standardized meal regimen was applied in this study: 20:30, a day before the study (recommendation): 100 g low-fat cottage cheese, 200 g oatmeal jelly); study day (served): 8:30: two eggs, unsweetened tea; 14:30: 100 g chicken breast, 100 g buckwheat porridge.

4.4. Salivary Melatonin Assessment

Fifteen volunteers were engaged in a standardized in-laboratory study [19]. Controlled lighting was used with lights-on (400 lx) from the start (10:00) until 18:00 and lights-off (<5 lx) from 18:00 to 08:00. A standardized water and food protocol was followed during these 26 h. Precautions were followed according to procedures for dim light melatonin onset (DLMO) measurements in saliva protocol [77]. Saliva samples were taken around the clock starting at 14:00 (at 14:00, 17:00, 20:00, 22:00, hourly from 22:00 to 04:00, at 06:00 and 10:00). The protocol is described in more detail elsewhere [19].

4.5. Genotyping

Genotyping was always performed by the same operator, who was not aware of the participant's clinical characteristics. Saliva samples were collected via standard protocols from nineteen patients as described elsewhere [17,19]. Polymorphic gene variants were identified by SNP (single nucleotide polymorphism) Screen Kit (Syntol; Moscow, Russia) for eight genes (clock genes: *PER2* rs6431590, *PER3* VNTR, *CLOCK* rs1801260 3111T/C, cryptochrome *CRY1* rs12820777; melatonin receptor genes *MTNR1A* rs34532313, *MTNR1B* rs10830963, G-protein *GNB* rs5443; and angiotensin converting enzyme, *ACE* rs1799752 insertion/deletion). In each reaction, two allele-specific hybridizations were used to detect two alleles of the studied polymorphism, independently on two fluorescence channels (ROX and FAM).

4.6. Data Analysis

Multiple linear regression and stepwise forward and backward analyses were applied to examine associations between serum lipids and factors related to glaucoma progression. One-way analyses of variance (ANOVA) tests for statistical differences were performed using the software packages Excel, STATISTICA 6, and SPSS 23.0. Shapiro–Wilk's W-test was applied to check for normal distribution. When variables were normally distributed (W-test's p-value > 0.05), a one-way ANOVA was used, with Tukey's post hoc correction for multiple testing. Otherwise, the Kruskal–Wallis and the Mann–Whitney post hoc tests were used. Statistical comparison of correlation strength was performed with cocor free online software [78]. The level of statistical significance was set at 5%.

5. Conclusions

Herein, we present evidence for a close association of RGC loss with changes in lipid metabolism in a time-dependent manner. Progressive loss of RGCs is linked to an HDL-C and TG increase in the morning, while TC and LDL-C have a strong evening gradient

and increase mainly in the evening (r = 0.533, $p < 0.0001$). A positive MEG (an increase in evening LDL-C) was particularly seen in a subset of POAG patients with the CLOCK_3111 TT genotype.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12136374/s1, Figure S1: No interaction between CLOCK_3111 TC polymorphism and glaucoma progression; F (1, 14) = 0.0018, $p = 0.967$.

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Informed Consent Statement: Written informed consent was obtained from all participants.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

A-POAG	Advanced primary open-angle glaucoma
GLV	Global loss volume
HDL-C	High-density lipid cholesterol
LDL-C	Low-density lipid cholesterol
MEG	Morning–evening gradient
POAG	Primary open-angle glaucoma
RNFL	Retinal nerve fiber layer
RGCs	Retinal ganglion cells
TC	Total cholesterol
TG	Triglycerides
SAP	Standard automated perimetry
SNP	Single nucleotide polymorphisms
S-POAG	Stable primary open-angle glaucoma

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