Quantitative Measurement of Hard Exudates in Patients With Diabetes and Their Associations With Serum Lipid Levels

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Purpose. To describe a reproducible method of quantifying macular hard exudates (HEs) in diabetic maculopathy and determine the associations of HEs with systemic risk factors.

Methods. Patients with diabetes were recruited from a tertiary eye hospital in Melbourne, Australia. Total macular area covered by HEs (total HE area) and the distance from the foveal center to the nearest HE were measured in a semi-automated manner. Associations between HE parameters and diabetic complication risk factors were examined using multiple linear regression models.

Results. Of 593 participants (mean age 60.5-years old), 97 (16.4%) had HEs in at least one eye, due to diabetic maculopathy. The intraclass correlation coefficients (ICC) for inter- and intra-observer agreements were 0.982 and 0.999, respectively. Total HE area was positively associated with qualitative HE severity scale determined by photographic graders. The median of total HE area was 0.089 mm^2 (interquartile range, 0.027–0.246). The median distance between foveal center and the nearest HE was 791.1 μm (431.9–1385.4). After adjusting for age, sex, duration of diabetes, glycated hemoglobin, mean arterial pressure, diabetic retinopathy level, and use of lipid-lowering medication, low density lipoprotein (LDL) cholesterol ($P = 0.009$), and triglyceride levels ($P = 0.036$) were positively associated with total HE area. Higher triglyceride levels were associated with central involvement ($P = 0.023$).

Conclusions. Quantitative measurement of HEs in patients with diabetes is associated with lipid levels, and higher triglyceride levels are associated with a higher risk of central involvement. Quantitative information may be useful to monitor HE progression or treatment response in persons with diabetic maculopathy.

Keywords: diabetic macular edema, hard exudate, diabetes, serum lipid, quantitative measurement

Hard exudates (HEs) in the retina are one of the hallmark signs of diabetic retinopathy (DR) and, when present within the macular region, indicate the presence or previous existence of diabetic maculopathy. While most epidemiologic studies have focused on risk factors of DR or diabetic macular edema (DME), several studies have reported specific associations between systemic factors and HEs, such as higher blood pressure, anemia, proteinuria, glycated hemoglobin (HbA1c), insulin use, and peripheral vascular disease.[1–4] Of these, elevated serum lipids have been shown to be consistently associated with presence of HEs.[5–7] A large amount of HEs in the macula characterizes a clinical diabetic maculopathy subtype that is especially difficult to treat, and can lead to substantial vision loss.[6]

Previous studies investigating the associations of HEs with systemic factors have assessed HEs qualitatively, using either presence or absence, or simple categorization of severity.[1,2,6,7] For example, Chew et al.[6] defined severity of HEs semiquantitatively using six grades (none, questionable, definite, obvious, moderate, and severe), and showed that HE severity was significantly associated with subsequent decrease in visual acuity in 5 years.[6] Although attempts to assess HEs quantitatively using imaging techniques have been proposed recently,[8,9] they are still at a developmental phase and have not been used in clinical settings. It is possible that quantitative measures of HEs may reveal better associations with systemic risk factors and, thus, help to improve our understanding of HEs. This could also potentially lead to an improved assessment and categorization of diabetic maculopathy severity based on the quantity of HEs present. This may be utilized as a guide to decide interventions to the associated conditions such as high serum lipid levels.

In this study, we describe a semi-automated method to quantitatively measure HEs in the macula from digital fundus photographs of patients with diabetes. We assessed the reproducibility of the proposed method, and report the associations of HEs with known risk factors of DR and diabetic maculopathy.

METHOD

Study Population

Data from this study were derived from participants of the Diabetes Management Project (DMP), and details of this study had been described elsewhere.[10] In brief, 613 English-speaking adults with diabetes, aged 18 years and older, were recruited from general and specialized eye clinics at the Royal Victorian Eye and Ear Hospital (Victoria, Australia) from March 2009 to July 2010. Of these, 14 patients were excluded due to suboptimal fundus image quality (i.e., poor focus, lashes, uneven illumination, etc.). In addition, six cases were excluded because of confounding or overlaying pathologies, namely, two patients had bilateral vitreous hemorrhage, and another four patients had chorioretinal atrophy from extensive laser photocoagulation covering the macular area. Of the remaining 593 subjects, 97 (16.4%) patients were found to have HEs and, thus, were included in this analysis.

Written, informed consent was obtained from all DMP participants. The study was approved by the Human Research and Ethics Committee of Royal Victorian Eye and Ear Hospital (08/815H) and adhered to the tenets of the Declaration of Helsinki.

Quantitative Measures of Hard Exudates

We used a public domain imaging software, ImageJ (version 1.44p; available in the public domain at http://rsb.info.nih.gov/ij//; National Institutes of Health, Bethesda, MD).[11,12] We measured two characteristics of HEs: total area covered by HEs and distance between the nearest HE and foveal center. These measurements were performed in a semi-automatic manner with minimal input from observers. In brief, digital retinal images of the right eye were used if both eyes were affected, while the left eye was used if the right eye image was not available. First, we measured the diameters of optic disc of all images (DD, in pixels) as a reference, given the likely differences in magnification of the retinal image due to differences in axial length, corneal curvature, and refractive error of different eyes. Second, the circular area within 3000 μm in radius from the fovea was extracted from the retinal color image. The extracted images were then split into three color channels of red, green, and blue. Green channel was used for the analysis because HEs and other retinal pathologies appear to be better contrasted in green channel than in the other two color channels.[13,14] Total area covered by HEs was extracted as the areas identified over the threshold of intensity using an automatic threshold function ("MaxEntropy" function in ImageJ software). When an area without HEs was detected over the threshold, or in the case of other pathologies that could potentially affect the detection of HEs, observers were allowed to eliminate such areas manually. If it was difficult to identify the precise areas covered by HEs, observers consulted an ophthalmologist and decided whether to exclude the area, and whether to call it "ungradable." Finally, the total area covered by HEs (total HE area) was measured automatically using the measure function; the results were then calculated relative to the average diameter of an optic disc (1800 μm[15]), and expressed in micrometers for distance and millimeters squared for total area (Fig. 1). The distance from the fovea to the nearest HE was also assessed according to the HEs' location, corresponding to the three concentric circles within 500-, 1500-, and 3000-μm radii from the foveal center. If the nearest HE was present within the 500-μm radius circle, it is considered to be "HEs involving central macula."

Inter- and Intra-Observer Reproducibility Assessment

Inter-observer agreement on total HE area was assessed using 97 images. Intra-observer agreement on estimation of total HE area was assessed using 40 randomly sampled images with repeated measurements of total HE area by the same observer 6 months later.

Comparison of Quantitative Assessment Against Qualitative Assessment

The total HE area measured semi-automatically in this proposed method was compared against subjective human grading of HE severity following the severity scales[6] using the Early Treatment Diabetic Retinopathy Study (ETDRS) standard photographs[16,17]; the severity was categorized as "None" = no HEs present; "Questionable" = questionable HEs present; "Definite" = definite HEs but less than ETDRS standard photograph 3; "Obvious" = HEs greater than or equal to ETDRS standard photograph 3, but less than standard photograph 5; "Moderate" = HEs greater than or equal to standard photograph 5, but less than standard photograph 4; "Severe" = HEs greater than or equal to standard photograph 4; and "cannot grade."

Assessment of Diabetic Macular Edema

DME was graded from fundus photographs based on the modified ETDRS DR severity scale,[16] and was considered present if there were visible retinal thickening or HEs in the macular area. If present, DME was further classified using a standard grid of the modified Wisconsin age-related maculopathy grading system,[18] as mild (retinal thickening or HE distant from the macula: between 1500 and 3000 μm from foveal center), moderate (retinal thickening or HE approaching the center of the macula: between 500 and 1500 μm from foveal center), or severe (clinically significant macular edema, defined as retinal thickening or HE in the center of the macula within 500 μm of foveal center).

Optical coherence tomography (OCT) was used to measure macular thickness and volume (Stratus Model 3000; Carl Zeiss Meditec Inc., North Ryde, New South Wales, Australia). Fast macular scans with retinal map analyses were performed for both eyes. Two measurements were recorded: inner macular thickness (IMT), defined as the mean retinal thickness within 3 mm diameter surrounding the central circular zone, and total macular volume (TMV), defined as the total volume within 6 mm diameter surrounding the central circular zone.

Blood and Urine Chemistry

Fasting (>8 hours) blood samples were collected for analysis of blood glucose, HbA1c, and lipids (total, high density lipoprotein [HDL], and low density lipoprotein [LDL] cholesterol and triglycerides). Fasting plasma glucose and serum lipids were estimated with a chemistry analyzer (Modular P; Roche Diagnostics, Mannheim, Germany). The Friedenwald formula was used to calculate LDL cholesterol where plasma triglyceride concentrations were less than or equal to 4.5 mM/L (400 mg/dL). In 17 participants with triglyceride concentrations

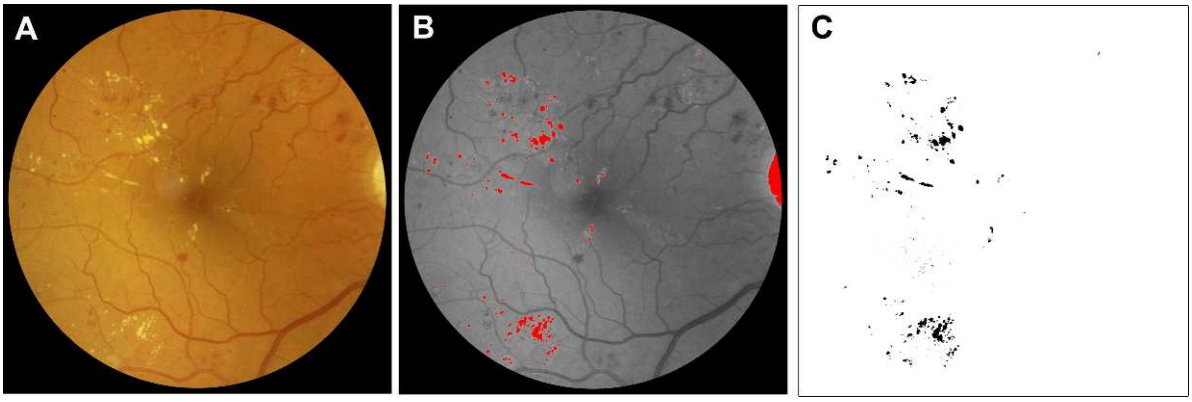

FIGURE 1. Quantitative assessment of the area covered by hard exudates. (**A**) Color fundus photograph cropped for 6000 μm-diameter square areas including black merging. (**B**) Area covered by HEs was identified with over threshold using automatic thresholding function (*red color area*). (**C**) The total area covered by HEs was extracted and measured automatically (the area corresponding to the optic disc area was eliminated manually).

greater than 4.5 mM/L, LDL cholesterol measurements were not calculated. A midstream urine sample was collected in 50 mL specimen containers to determine the albumin–creatinine ratio (ACR). All blood and urine analyses were performed at Melbourne Pathology (Melbourne, Australia), with individual results electronically delivered through a password-protected program. The laboratory is accredited to the International Standard ISO15189 (Medical Laboratories) and is certified by National Association of Testing Authorities (NATA).

Assessment of Other Risk Factors

Each participant underwent a comprehensive assessment that included a range of clinical, biochemical, and anthropometric measurements, and lifestyle factors collected from validated questionnaires. Key covariates included age, sex, duration of diabetes (years), mean arterial pressure (systolic blood pressure × 1/3 + diastolic blood pressure × 2/3), diabetic medication use, use of antihypertensive medications, use of lipid-lowering medications, HbA1c level (percentage), blood pressure (mm Hg), body mass index (BMI; kilograms divided by height in meters squared), waist circumference (WC; in centimeters), ACR, and smoking status.

Statistical Analysis

Reproducibility of the method was assessed using intraclass correlation coefficient (ICC) for inter- and intra-observer treatments, and Bland-Altman agreement for interobserver agreements. The association between total HE area estimated using this new method and HE severity determined following ETDRS standard photographs[6,16,17] was assessed using multiple linear regression models. The associations between quantitative measure of total HE area and serum lipid levels and other systemic characteristics were assessed using multiple linear regression models without and with adjusting for age, sex, duration of diabetes, HbA1c, mean arterial pressure, DR severity, and use of lipid-lowering medication. Association estimates (beta coefficients) from these models are expressed as per unit change in the study factor associated with millimeters squared changes in total HE area. The associations between having HEs involving the central macula and serum lipid levels and other systemic characteristics were assessed using logistic regression models with and without adjusting for age, sex, duration of diabetes, HbA1c, mean arterial pressure, DR severity, and use of lipid-lowering medication. Association estimates (odds ratios) of the logistic regression models are expressed as per unit change in the study factor associated with odds of having HEs in central location. The association between total HE area and both the photograded severity of DME and OCT measurements were assessed using linear regression models. The association between HEs involving central macula and visual acuity (VA) was assessed using multiple linear regression models.

RESULTS

Table 1 indicates characteristics of the diabetic patients with HEs. Of 97 patients with HEs, the median total HE area was 0.089 mm^2 (interquartile range, 0.027–0.246). Median distance between the fovea and the nearest HE was 791.1 μm (431.9–1385.4). ICCs for inter- and intra-observer agreement on total HE area estimates were 0.982 and 0.999, respectively, indicating the method to be highly reproducible. Bland-Altman agreement is shown in Figure 2; the mean difference was 0.016 mm^2 and 95% limits of agreement were –0.129 to 0.160.

Comparison of total HE area measured using quantitative semi-automatic method with the qualitative HE severity levels[6] is shown in Figure 3. Ninety-seven fundus images with HEs were graded as "definite" *n* equals 7, "obvious" *n* equals 30, "moderate" *n* equals 32, and "severe" *n* equals 28. Total HE area was significantly and positively associated with greater HE severity levels ($P = 0.001$). Only one case with HEs as categorized as definite could not be detected as having HEs by the quantitative assessment.

In crude analysis, total cholesterol levels were positively associated with total HE area (for each 1 mM/L increase, mean change in total HE area: 0.097 mm^2, 95% confidence interval [CI]: 0.042–0.152; Table 2). Similarly, higher LDL cholesterol levels were positively associated with total HE area (mean change in total HE area 0.105 mm^2, 95% CI 0.039–0.172). Each 1 mM/L increase in triglycerides was associated with 1.90 times the odds of having HEs involving the central macula ($P = 0.015$) (Table 2).

After adjusting for age, sex, duration of diabetes, HbA1c, mean arterial pressure, DR severity, and use of lipid-lowering medication, LDL cholesterol and triglyceride levels were positively associated with total HE area (Table 3). Each 1 mM/L increase in triglycerides was associated with 2.10 times the odds of having HEs involving the central macula ($P = 0.023$; Table 3). Other systemic risk factors, such as HbA1c and ACR were not significantly associated with HE parameters (data not shown).

Total HE area was not associated with OCT parameters (IMT or TMV). There was a marginally nonsignificant trend of

Table 1. Participants Characteristics of Those With HEs Detected in the Macular Area ($N = 97$)

Characteristics	Mean (±SD)/Median (Interquartiles)*
Age, y	60.5 (±9.96)
Sex, male %	70.9
BMI, kg/m^2	31.1 (±6.09)
Type of diabetes, type 2 %	90.4
Duration of diabetes, y	16.7 (±8.6)
Use of insulin, %	55.3
Haemoglobin A1c, %	8.53 (±1.53)
Systolic blood pressure, mm Hg	141.9 (±20.6)
Diastolic blood pressure, mm Hg	77.9 (±9.07)
Urine microalbumin, mg/L	46.5 (12–364)
ACR, mg/mM	6.35 (1.2–47.8)
Total cholesterol, mM/L	5.04 (±1.42)
Triglycerides, mM/L	1.7 (1.2–2.3)
HDL cholesterol, mM/L	1.43 (±0.91)
LDL cholesterol, mM/L	2.38 (±1.22)
Total HE area, mm^2	0.089 (0.027–0.246)
Distance between the fovea and the nearest HE, μm	791.1 (431.9–1385.4)

* Data are mean (±SD) for normally distributed data, or median (interquartile ranges) for skewed data unless otherwise stated.

increasing total HE area associated with severer levels of DME ($P = 0.059$), graded using color photographs. Microangiopathy such as the presence of retinal hemorrhages or microaneurysms in the macula was not associated with total HE area (data not shown).

The presence of HEs involving the central macula was associated with +0.181 increase in logMAR pinhole VA (i.e., poorer VA) than those without central macula involvement of HEs ($P = 0.045$). There was no association between total HE area with logMAR pinhole VA.

Discussion

In this study, we employed a semi-automated protocol to quantify two parameters related with HEs. This method displayed high inter- and intra- observer agreements. We also showed that total HE area was associated with the severity of HEs as assessed manually by photographic graders using ETDRS standard photographs. We found that after adjusting for age, sex, duration of diabetes, HbA1c, mean arterial pressure, DR severity, and use of lipid-lowering medication, higher LDL cholesterol and triglyceride levels were independently associated with increased total HE area in adult patients with diabetes. Patients with higher triglyceride levels were more likely to have HEs in the central macular region. Severity of DME graded using color photographs was not significantly associated with total HE area (P for trend = 0.059). Central macular involvement of HEs was associated with poorer vision.

The main purpose of the quantitative assessment method is to provide clinicians a simple and precise quantity of HEs, which is a useful objective measure when analyzing the association of HEs with systemic factors or other eye conditions. OCT enabled us to quantify edema in diabetic maculopathy, whereas there has not been a standardized method to quantify HEs, an important component of diabetic maculopathy. Quantitative measures of HE may have a potential to be used as a sensitive surrogate marker for severity levels of diabetic maculopathy, and could potentially be used in clinical trials or studies assessing diabetic maculopathy treatment. This is particularly advantageous in studies with a relatively short period of follow up, as qualitative assessment of HE using categorical severity levels may not be sensitive enough to capture subtle changes in HE area and location. We demonstrated that total HE area was closely associated with the

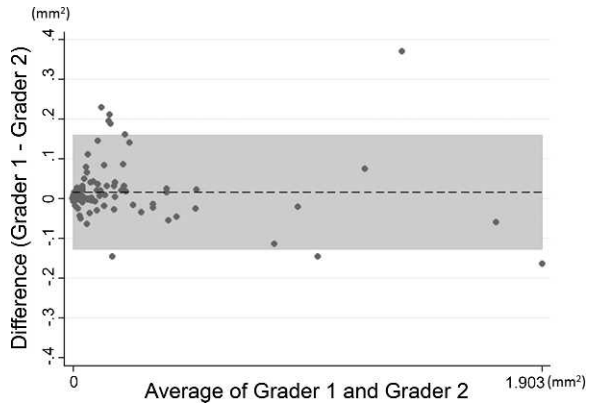

Figure 2. Bland Altman agreement with two observers. The mean difference was 0.016 mm^2 and 95% limits of agreement were −0.129 to 0.160.

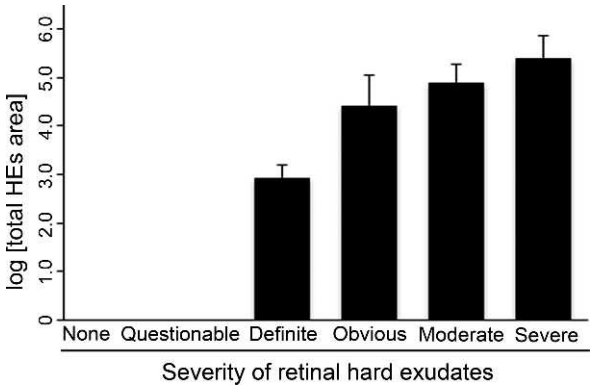

Figure 3. The association of the severity levels of HEs between ETDRS and our procedure. "None" = no HEs present; "Questionable" = questionable HEs present; "Definite" = definite HEs but less than ETDRS standard photograph 3; "Obvious" = HEs greater than or equal to ETDRS standard photograph 3, but less than standard photograph 5; "Moderate" = HEs greater than or equal to standard photograph 5, but less than standard photograph 4; "Severe" = HEs greater than or equal to standard photograph 4.

Table 2. The Univariate Association Between Systemic Risk Factors and HE Parameters

	Total HE Area, mm²		HEs Involving the Central Macula, ≤500 μm	
Systemic Characteristics	**Mean Difference (95% CI)**	***P***	**Odds Ratio (95% CI)***	***P***
Total cholesterol, per 1 mM/L	0.097 (0.042, 0.152)	0.001	1.05 (0.78, 1.39)	0.760
LDL cholesterol, per 1 mM/L	0.105 (0.039, 0.172)	0.002	0.91 (0.64, 1.29)	0.607
HDL cholesterol, per 1 mM/L	0.019 (−0.071, 0.109)	0.573	1.45 (0.78, 2.69)	0.243
Triglycerides, per 1 mM/L	0.054 (−0.033, 0.141)	0.221	1.90 (1.13, 3.18)	0.015
Duration of diabetes, per 1 y	0.007 (−0.017, 0.003)	0.166	0.93 (0.88, 0.98)	0.012
Haemoglobin A1c, per 1%	0.055 (−0.001, 0.112)	0.056	0.91 (0.69, 1.20)	0.507
Use of insulin	−0.144 (−0.313, 0.026)	0.097	1.09 (0.49, 2.44)	0.834
Use of antihypertension medication	−0.064 (−0.024, 0.110)	0.464	1.21 (0.52, 2.83)	0.652
Use of lipid-lowering medication	−0.051 (−0.248, 0.147)	0.611	0.79 (0.30, 2.07)	0.631

* Eyes with HEs involving the central macula (versus eyes with HEs involving the inner or outer circle).

qualitative severity scale determined by graders for validation purpose. Here, only one case in the definite category could not be detected as having HEs by proposed qualitative semi-automatic method. We speculate that this is because that the amount of HEs in the ETDRS standard photograph 3 is very mild and subtle; this warrants further evaluation of our proposed method to increase the sensitivity of HE detection, although the clinical significance of quantifying very subtle HEs is uncertain.

Previous clinical and epidemiologic studies have examined the associations between systemic factors and HEs using qualitative assessment methods.[1,2,6,7] HEs have been used as a marker of past or present diabetic maculopathy to determine the effect of lipid-lowering medications in treating diabetic maculopathy.[19–21] Some clinical trial studies have assessed the severity of HEs using ETDRS standard photographs as a guide,[21] and others have adopted the standard macular grid/circles used to estimate drusen size and area for AMD.[16] We have applied a quantitative method to assess HEs in this study. Quantitative measures of area and location of HEs have an advantage over subjective, qualitative assessments in describing changes in quantity of HEs objectively and precisely. HEs are usually small and widely scattered deposits, and therefore subjective assessments may not be able to detect subtle changes over time. Although methods of detecting HEs by imaging techniques have been explored,[8,9] these have not routinely been used in clinical settings.

The ImageJ software (National Institutes of Health) is designed to measure the area or distance in pixels with various thresholding methods. This method is fast (average time to measure total HE area approximately 45 seconds) and we were able to achieve high inter- and intra-observer agreement due to limited manual input from observers. The simplicity of the protocol may allow clinicians to apply it for both clinical and research purposes.

Roy and Klein[2] reported that the presence of HEs was associated with sex, diastolic blood pressure, proteinuria, HbA1c, and peripheral vascular disease in 725 African Americans with type I diabetes. Several studies found a relationship between the presence of HEs and blood pressure[1,4] and renal function,[2] while others did not replicate these associations.[3] On the other hand, serum lipid levels such as total and LDL cholesterol have been consistently found to be associated with HEs.[5–7] The association of total HE area with LDL cholesterol, observed in our study sample, is consistent with previous studies.[5–7] However, among serum lipids, the reported association between HEs and triglycerides has been inconsistent[5] or controversial.[1,4] Chew et al.[6] reported that serum triglyceride level was not associated with the presence of HEs, but was associated with a more rapid onset of the obvious category of HEs. The associations of total HE area and central location of HEs with triglycerides, found using our newly developed quantitative HEs measurement method, needs confirmation in future studies.

It has been reported that HE severity was associated with worse visual outcomes in patients followed over 5 years.[6] The vision was affected by the presence of HEs in fields close to central macula in a previous study.[16] The presence of HEs was also associated with reduced retinal sensitivity on microperimetry.[22] In this study, we confirmed that involvement of the central macular region (within 500-μm radius) was associated with poorer VA, while total HE area was not associated with VA. Due to the fact that not only the severity but also the location of HEs can affect vision, we consider the distance of HEs from the foveal center to be an important determinant of visual function.

The strengths of our study include a well-characterized clinical sample of diabetic patients with various levels of HEs related with diabetic maculopathy, the use of standardized assessment to define DR and DME by trained graders.[23] However, there are limitations. First, the cross-sectional nature of the study does not allow us to assess the temporal sequence of these associations. Second, quantification of HEs could have been affected by retinal characteristics such as chorioretinal color, fundus tessellation in high myopia, mild chorioretinal atrophy, and severe scaring after retinal laser photocoagulation,

Table 3. The Multi-Adjusted Associations Between Serum Lipid and HEs

	Total HE Area, mm²		HEs Involving the Central Macula, ≤500 μm	
	Mean Difference (95% CI)	***P***	**Odds Ratio (95% CI)***	***P***
LDL cholesterol, per 1 mM/L	0.110 (0.028, 0.191)	0.009	0.67 (0.42, 1.07)	0.095
HDL cholesterol, per 1 mM/L	0.052 (−0.155, 0.260)	0.616	1.51 (0.49, 4.72)	0.474
Triglycerides, per 1 mM/L	0.112 (0.007, 0.217)	0.036	2.10 (1.11, 3.97)	0.023

Adjusted for age, sex, duration of diabetes, hemoglobinA1c, mean arterial pressure, diabetic retinopathy level, and use of lipid lowering medication.

* Eyes with HEs involving the central macula (versus eyes with HEs involving the inner or outer circle).

although these confounders were mitigated by manual observer input. There is a need to improve imaging techniques to quantify HEs in eyes with minimal HEs or with various backgrounds. Finally, to calibrate magnification of retinal images, the disc-to-macula distance is a more precise calibration factor[24] than the method we used in this study. In cases where there are no HEs accumulated in the fovea, the disc-to-macula distance should be used and will provide more precise estimation of lesion areas.

In conclusion, we employed a simple and reproducible method to quantify HEs in patients with past or present diabetic maculopathy, and found that higher serum LDL cholesterol and triglyceride levels were associated with greater total area of HEs. Also, triglyceride levels were associated with the central location of HEs. Additional quantitative information may improve the ability to follow the clinical progression of HEs or their response to the treatment in persons with diabetic maculopathy. This may be utilized as a marker of the severity and activity to monitor changes of diabetic maculopathy associated with treatment.[20,25] Prospective studies are needed to clarify the temporal relationship between serum lipids and total area and location of HEs, and evaluate the potential of this quantitative measure in clinic practice.

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