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Original Article

Retinal microvascular dysfunction in hypercholesterolemia

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Retinal vessel analysis;
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Hypercholesterolemia;
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BACKGROUND: Hypercholesterolemia is one of the most important contributors to atherosclerosis. Whether hypercholesterolemia also affects the retinal microcirculation is unclear.

OBJECTIVE: The goal of our study was to assess the association of cholesterol levels with retinal microvascular function using dynamic and static retinal vessel analysis (RVA) in a primary prevention setting.

METHODS: This cross-sectional, observational study prospectively recruited 67 patients with hypercholesterolemia without known cardiovascular disease (mean age 64.4 ± 10.4 years; 45% female) and 78 healthy controls (mean age 61.8 ± 11.2 years; 45% female). The primary end point of the study was flicker-induced dilatation of retinal arterioles (FID_{art}) with secondary exploratory outcomes including venular FID (FID_{ven}), arteriovenous ratio, flow-mediated dilatation and arterial stiffness as measured with augmentation index and pulse wave velocity. Multiple regression analysis was performed to study the association of cholesterol levels with retinal microvascular function.

RESULTS: FID_{art} was significantly impaired in patients with hypercholesterolemia compared with healthy controls (mean FID_{art} 2.1 ± 1.8 vs 3.1 ± 1.8%, $P = .001$). This association remained when analysis was restricted to dyslipidemic patients without coexisting hypertension or lipid-lowering therapy. No significant differences remained for FID_{ven}, flow-mediated dilatation, arteriovenous ratio, or arterial stiffness between the groups. Low-density lipoprotein, but not high-density lipoprotein, cholesterol was a significant negative predictor of FID_{art} in multiple regression analysis.

CONCLUSION: Hypercholesterolemia is associated with significant retinal microvascular dysfunction as evidenced by a reduction in flicker-induced dilatation of retinal arterioles. Dynamic RVA may be a promising method for the study of retinal microvascular dysfunction in populations at elevated cardiovascular risk.

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Introduction

Hypercholesterolemia, elevated low-density lipoprotein (LDL) cholesterol in particular, represents one of the most important modifiable risk factors for atherosclerosis. An early phenomenon observed in the process of atherosclerosis is endothelial dysfunction, a state characterized by impaired vasodilatation, vessel wall integrity, and blood coagulation.[1] Hypercholesterolemia is also associated with endothelial dysfunction, which is potentially reversible with lipid-lowering therapy making its measurement attractive for primary prevention.[2]

While methods quantifying endothelial function in larger conduit vessels are well established, there is a lack of ways to measure endothelial function in the microcirculation. Retinal vessel analysis (RVA) is a novel and unique method allowing measurement of dilatation of small retinal arterioles and venules in response to flicker light using high-resolution fundus videography.[3] This process has been found to involve nitric oxide (NO) signaling, and thus, impairment in retinal vasodilatation may be a new and attractive noninvasive biomarker for microvascular endothelial dysfunction.[4]

Systematic studies on flicker-induced retinal vasodilatation in hypercholesterolemia, especially in the primary prevention setting are still scarce.[5,6] It was therefore the goal of this study to evaluate the extent of retinal microvascular dysfunction in a cohort of patients with hypercholesterolemia but without known cardiovascular disease compared with healthy controls.

Methods

Study design and protocol

The study had an observational cross-sectional design with prospective recruitment of patients and controls for investigation of vascular function with focus on the retinal microvasculature. The study protocol was approved by the local ethics committee as part of a larger investigation of retinal microvascular function in different disease groups (KEK-ZH-No. 2014–0329).[7] All participants provided written informed consent. For this study, 2 groups of patients were recruited: 1. patients with hypercholesterolemia defined as either preexisting treatment with a lipid-lowering drug or untreated patients with an LDL cholesterol of 4.1 mmol/L (159 mg/dL) or greater (high risk group in primary prevention),[8] 2. healthy controls (HC) defined as participants without known cardiovascular risk factors or diseases. Exclusion criteria for the study's participants were age under 40 years (to achieve better matching of age between volunteers and patients), preexisting cardiovascular disease (including coronary artery disease, peripheral artery disease, heart failure, or stroke), diabetes mellitus, smoking, pregnancy or breastfeeding, allergy against study drugs, photosensitive epilepsy, glaucoma, or other significant eye pathologies such as blindness, inability to fixate, or prior retinal laser coagulation. Patients and controls were recruited directly at the outpatient unit of the University Heart Center Zurich or using advertisements in sports and elderly clubs in the greater Zurich area.

After signing informed consent, participants were invited to the primary study visit, which was conducted in the morning and included a medical history, measurement of clinical and laboratory parameters, as well as vascular function assessments (arterial stiffness and RVA, followed by flow-mediated vasodilatation at the end of the examination). Patients were instructed to remain fasted for at least 8 hours (except water), take their regular medication as planned, refrain from coffee or alcohol consumption for at least 12 hours, avoid unusual exercise the day before the examination, and only present in stable medical state (ie, free of infections or acute illnesses). Patients and controls were recruited in parallel during the same period and studied sequentially based on available time slots.

Retinal vessel analysis

RVA was conducted using an Imedos Dynamic RVA (Imedos, Jena, Germany) that consisted of a Zeiss FF450 plus fundus camera (Carl Zeiss Meditec AG, Jena, Germany) connected with 2 charge-coupled device cameras that provide digital images for a computerized vessel analysis software (Imedos, Jena, Germany). Previously established and validated protocols where used in this study.[3,9] In brief, 1 eye was randomly selected and mydriasis was induced using 0.5% tropicamide eye drops. After 20 minutes, dynamic RVA was conducted with measurement of dilatation of retinal arterioles and venules in response to 12.5 Hz optoelectronic flicker light stimulation. Analysis was performed on temporal segments of 1 retinal arteriole and venule 0.5 to 2 optic disc diameters away from the optic disc. The selected vessel segments had to be sharp, free of branching sites and reflex phenomena, and supplied at least 1 branch arteriole to the macula region. The measurement consisted of a 50 seconds baseline and three 20 seconds flicker stimulations each followed by a recovery period of 80 seconds. The results from the 3 flicker periods were averaged and percent dilatation of the arteriole or venule from baseline (FID_{art} and FID_{ven}, respectively) was calculated automatically using Imedos analysis software. Reproducibility of the method and the used measurement protocol has been demonstrated before.[10] The authors report very good intraclass and intraobserver correlations (ICC 0.82), with 18% of the variability in measurements of FID_{art} due to errors in the measurement process and the observer. No significant bias was found in Bland-Altman analysis (−0.089, CI95 [−1.85; +2.02], no measurement outside limits of agreement). For static RVA, monochromatic 50° fundus photographs were recorded using Visualis and VesselMap 2 software (Imedos, Jena, Germany). Retinal artery and vein diameters in the area 0.5 to 1 optic disc diameters

distant from the optic disc were summated for calculation of the central retinal artery and vein equivalent (CRAE and CRVE).[9] CRAE and CRVE are plotted in relative measuring units (mu). Both values were used to calculate the arteriovenous ratio (AVR = CRAE/CRVE).

Flow-mediated vasodilatation

Flow-mediated dilatation (FMD) was measured using established protocols.[1] In short, arterial diameter of 1 brachial artery was continuously measured using a 10-MHz linear array transducer (Siemens Acuson X300, Siemens AG) with automatic wall-tracking and analysis software (FMD-Studio, Pisa, Italy). One minute after its application to the lower arm, the blood pressure (BP) cuff was inflated 50 mm Hg above systolic pressure for 5 minutes. After release, hyperemia occurred and the change in arterial diameter was measured for another 10 minutes. The percent maximum dilatation related to the baseline diameter was calculated and shown as FMD (%). To determine endothelial-independent effects, pharmacological peak percent dilatation of the brachial artery was measured 6 minutes after 1 dose of sublingual glycerol trinitrate (Nitrolingual 0.4 mg, Pohl-Boskamp, Germany). The reproducibility of our laboratory's measurements was published previously.[11]

Arterial stiffness

Arterial stiffness was measured with a SphygmoCor applanation tonometer (AtCor Medical, Itasca, IL) according to established protocols.[1,12,13] In brief, patients rest in the supine position for 15 minutes and measurements are taken immediately after brachial BP recording. Augmentation index (AIX) was measured at the level of the radial artery by obtaining 10 high-quality pulse wave measurements with automatic calculation of AIX using the manufacturer's proprietary software and after normalizing to a heart rate of 75 beats per minute. Pulse wave velocity (PWV) was calculated from the pressure wave transit time and distance between carotid and femoral artery according to recent guidelines.[12,14,15]

Transit time between arterial sites was determined in relation to the R wave of the electrocardiogram.

Laboratory assessments

Blood samples were taken in the fasted state using heparin plasma vials at the beginning of the study visit and analyzed on the same day at the Institute of Clinical Chemistry, University Hospital Zurich with established methods. LDL cholesterol was determined using the Friedewald formula. Then 10-year risk of fatal cardiovascular disease was calculated using the European Society of Cardiology risk score for low-risk countries.[16] High-sensitivity troponin T was quantified using electrochemiluminescence-immunoassays and the COBAS8000 autoanalyzer of Roche Diagnostics (Mannheim, Germany). Undetectable values were replaced by half the lower limit of detection.[17]

Statistical analysis

Statistical analysis was performed with JMP 12.1 (SAS Institute, Cary, North Carolina). Figures were prepared using Graphpad Prism 5.0 (GraphPad Software, San Diego). The primary end point of the study was the difference in FID_{art} between patients with hypercholesterolemia and HC. The other vascular measurements (FID_{ven}, AVR, CRAE, CRVE, FMD, PWV, and AIX) were secondary exploratory outcomes. Results are expressed as mean ± standard deviation unless otherwise noted. Sample size was estimated based on data on FID_{art} of HC by Mandecka *et al.*[18] Estimating a difference of FID_{art} of 1% (standard deviation 2.1) with power of 80% and an alpha error of 5%, a group size of 70 patients was determined.

Normality was assessed visually using quantile-quantile plots. Categorical variables were analyzed using chi-square test or Fisher's exact test as appropriate. Comparison of 2 continuous variables with normal distribution was tested with Student's *t*-test for equal variances or Welch's test for unequal variances. Non-normally distributed data were tested with the Wilcoxon test. Three group comparisons were performed using 1-way analysis of variance as omnibus and Student's *t*-test post hoc. Multiple linear regression analysis was used to study the relationship of vascular function parameters with cholesterol levels and potential confounders. Based on previous data, age, body mass index, systolic BP, and presence of lipid-lowering or antihypertensive therapy, in addition to LDL cholesterol and high-density lipoprotein (HDL) cholesterol were included in the model.[6,19] All tests were 2-sided and a *P*-value of less than .05 was considered significant.

Results

Baseline characteristics

Between January 2015 and February 2017, 78 healthy controls (mean age 61.8 ± 11.2 years; 45% female) and 67 patients with hypercholesterolemia (mean age 64.4 ± 10.4 years; 45% female) met the eligibility criteria and were included in the study. All participants were of Caucasian origin. Their baseline characteristics are shown in Table 1. The mean total and LDL cholesterol in patients with hypercholesterolemia was 6.0 ± 1.3 and 3.8 ± 1.3 mmol/L (232 ± 50 and 147 ± 50 mg/dL), significantly higher than in HC with 5.2 ± 0.6 and 3.0 ± 0.6 mmol/L (201 ± 23 and 116 ± 23 mg/dL), respectively (both $P < .001$). No significant difference in HDL cholesterol was observed between patients with hypercholesterolemia and HC (1.7 ± 0.4 vs 1.8 ± 0.4 mmol/L [66 ± 15 vs 70 ± 15 mg/dL], $P = .17$). Patients with hypercholesterolemia had a significantly higher BP and a hypertension prevalence of 55%

compared with HC. The 10-year risk of fatal cardiovascular disease was significantly higher in patients with hypercholesterolemia compared with HC (European Society of Cardiology SCORE 2.9% ± 1.9 vs 2.1% ± 1.9, $P = .004$). There were no differences in the inflammation marker high-sensitivity C-reactive protein, thyroid function as measured by thyroid-stimulating hormone, kidney function (estimated glomerular filtration rate), as well as cardiac biomarkers (creatine kinase and high-sensitivity troponin T).

RVA and other vascular outcomes

RVA was performed and tolerated well in all participants except 1 healthy control due to intolerance of the flicker stimulation. Results of the vascular assessments are shown in Table 2 and Figure 1. There was a significant difference in the primary end point with lower FID_{art} in patients with hypercholesterolemia compared with HC (mean FID_{art} 2.1 ± 1.8 vs 3.1 ± 1.8%, $P = .001$). Among the secondary end points, patients with hypercholesterolemia had a significantly lower AVR compared with HC (0.82 ± 0.07 vs 0.84 ± 0.06, $P = .01$) and a higher arterial stiffness as measured by PWV (8.8 ± 2.4 vs 7.5 ± 1.7, $P < .001$). By contrast, there was no difference in FID_{ven}, CRAE, CRVE, FMD, glycerol trinitrate, and AIX_{HR75} between patients with hypercholesterolemia and HC. No correlation of FID_{art} with FMD was found ($r < 0.01$, $r^2 < 0.001$, $P = .95$). FID_{art} correlated weakly with AVR ($r = 0.18$,

Table 1 Baseline characteristics

Parameter	HC (n = 78)	Hypercholesterolemia (n = 67)	*P*-value
Clinical characteristics			
Age (y)	61.8 ± 11.2	64.4 ± 10.4	.14
Female sex (n)	35 (45%)	30 (45%)	.99
BMI (kg/m^2)	24.5 ± 3.6	25.5 ± 2.7	.07
Systolic BP (mm Hg)	126.4 ± 10.7	135.0 ± 15.5	**<.001**
Diastolic BP (mm Hg)	78.0 ± 8.1	83.3 ± 11.5	**.002**
Heart rate (bpm)	64.4 ± 10.1	66.5 ± 9.8	.21
ESC SCORE (%)	2.1 ± 1.9	2.9 ± 1.9	**.004**
Comorbidities			
Smoking	0 (0%)	0 (0%)	-
Diabetes mellitus	0 (0%)	0 (0%)	-
Hypertension	0 (0%)	37 (55%)	**<.001**
Laboratory parameters			
Total cholesterol (mmol/L)	5.2 ± 0.6	6.0 ± 1.3	**<.001**
HDL cholesterol (mmol/L)	1.8 ± 0.4	1.7 ± 0.4	.17
LDL cholesterol (mmol/L)	3.0 ± 0.6	3.8 ± 1.3	**<.001**
Triglycerides (mmol/L)	1.0 ± 0.4	1.3 ± 0.8	**.001**
Fasting plasma glucose (mmol/L)	5.2 ± 0.5	5.4 ± 0.5	.18
CRP, high sensitivity (mg/L)	1.6 ± 1.3	1.5 ± 1.7	.85
TSH (mU/L)	2.4 ± 1.2	2.3 ± 1.3	.76
eGFR CKD-EPI (mL/min)	85.8 ± 15.2	83.3 ± 14.6	.32
CK (U/L)	108.8 ± 82.2	109.2 ± 58.3	.97
Troponin T, high sensitivity (ng/L)	8.7 ± 5.0	8.2 ± 3.2	.66
Concomitant medication			
Statin	0 (0%)	29 (43%)	**<.001**
Ezetimibe	0 (0%)	3 (4%)	.10
Any antihypertensive drug	0 (0%)	25 (37%)	**<.001**
ACEI/ARB	0 (0%)	21 (31%)	**<.001**
Beta-blocker	0 (0%)	6 (9%)	**<.001**
Calcium channel blocker	0 (0%)	10 (15%)	**<.001**
Thiazide	0 (0%)	5 (7%)	**.02**
Aspirin	0 (0%)	14 (21%)	**<.001**
NSAID	2 (3%)	2 (3%)	1.00
Vitamin or mineral supplement (%)	23 (29%)	21 (31%)	.81

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; BP, blood pressure; CK, creatine kinase; CRP, C-reactive protein; eGFR CKD-EPI, estimated glomerular filtration rate as calculated by chronic kidney disease epidemiology collaboration formula; ESC, European Society of Cardiology; HC, healthy controls; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NSAID, nonsteroidal anti-inflammatory drugs; TSH, thyroid-stimulating hormone.

Statistical analysis of continuous variables: Student's t post test or Welch's test as appropriate.

Statistical analysis of categorical variables: chi-square test or Fisher's exact test as appropriate.

To convert cholesterol from mmol/L to mg/dL, multiply by 38.67.

To convert trigylcerides from mmol/L to mg/dL, multiply by 88.57.

Significant differences between the groups ($P < .05$) are shown in bold.

$r^2 = 0.03$, $P = .04$). On the other hand, a stronger negative correlation existed between AVR and PWV ($r = -0.3$, $r^2 = 0.09$, $P < .001$).

Subgroup analyses and multiple regression

Because of the established effects of hypertension on the retinal vasculature and a possible modulation of vascular function by lipid-lowering therapy, a subgroup analysis was performed in hypercholesterolemia patients without hypertension and with (n = 8, mean LDL cholesterol 3.0 ± 0.8 mmol/L [116 ± 31 mg/dL]) or without lipid-lowering therapy (n = 22; mean LDL cholesterol 5.0 ± 1.0 mmol/L [193 ± 39 mg/dL]). There were no significant differences in systolic BP between these subgroups (127 ± 8 mm Hg in dyslipidemic subjects without lipid-lowering therapy, 125 ± 19 mm Hg in dyslipidemic subjects with lipid-lowering therapy and 126 ± 11 mm Hg in HC, all $P > .05$).

Normotensive subjects with untreated hypercholesterolemia had a significantly lower FID_{art} than HC (1.5 ± 1.3 vs 3.1 ± 1.8%, $P < .001$; Fig. 2A). No significant differences in FID_{art} were seen between patients with treated hypercholesterolemia and HC. By contrast, AVR and PWV were not significantly different between these normotensive subgroups (Fig. 2B, C, respectively).

To determine whether the association of LDL cholesterol with retinal microvascular function was modified by potential confounders, multiple linear regression analysis was performed (Table 3). Respective univariate regressions are shown in Supplementary Figure 1. LDL cholesterol remained the strongest negative predictor of FID_{art} (standardized $\beta = -0.25$, $P = .007$) in the multivariable model ($F(7,143)$ ratio = 2.2, $r^2 = 0.10$, r^2 adj = 0.06, $P = .04$). A negative association was also observed for FID_{art} with age (standardized $\beta = -0.19$, $P = .03$), while no significant associations with body mass index, systolic BP, lipid-lowering or antihypertensive drugs, or HDL cholesterol were found. Concerning the secondary vascular outcomes, no significant associations with LDL cholesterol were found (Supplementary Table 1). Systolic BP was significantly associated with AVR, CRAE, PWV, and AIX_{HR75} in the multivariable model.

Discussion

In this study of RVA in hypercholesterolemia in the primary prevention setting, FID_{art}, a marker of retinal microvascular function, was significantly reduced in patients with hypercholesterolemia compared with HC. This association was also observed when analysis was limited to patients without coexisting hypertension or lipid-lowering therapy. In multivariable analysis, LDL cholesterol remained a significant negative predictor of FID_{art}.

While the detrimental effects of hypertension and diabetes on the retinal vasculature are well established and studied via funduscopy, less is known on the association of hypercholesterolemia with retinal microvascular function. In this study, we showed that elevated LDL cholesterol is significantly associated with reduced flicker-induced retinal arteriolar dilatation, independent of BP and other potential confounders. Our results are in line with 2 smaller studies.[5,6] Pemp *et al.*[6] studied dynamic RVA in 40 patients with hypertension and/or elevated cholesterol and showed that FID_{art} is significantly reduced in this population compared with controls. They also noted a significant difference in FID_{ven}. However, the groups were not matched for age and sex, multivariate analysis controlling for BP was not performed, and measurement of LDL and HDL cholesterol was not

Table 2 Vascular assessments in patients with hypercholesterolemia and controls

Parameter	HC (n = 78)	Hypercholesterolemia (n = 67)	*P*-value
Retinal vessel analysis			
FID_{art} (%)	3.1 ± 1.8	2.1 ± 1.8	**.001**
FID_{ven} (%)	4.5 ± 2.2	4.2 ± 2.0	.46
AVR	0.84 ± 0.06	0.82 ± 0.07	**.01**
CRAE	181.0 ± 15.6	176.8 ± 16.8	.13
CRVE	214.8 ± 17.0	216.7 ± 16.9	.51
Flow-mediated vasodilation			
FMD (%)	5.9 ± 3.4	5.6 ± 2.7	.50
GTN (%)	17.0 ± 5.8	16.2 ± 6.1	.54
Arterial stiffness			
PWV (m/s)	7.5 ± 1.7	8.8 ± 2.4	**<.001**
AIX_{HR75} (%)	26.1 ± 11.1	25.9 ± 9.0	.89

AIX_{HR75}, augmentation index normalized to heart rate of 75/min; AVR, arteriovenous ratio; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; FID_{art}, flicker-induced dilatation of retinal arterioles; FID_{ven}, flicker-induced dilatation of retinal venules; FMD, flow-mediated vasodilatation; GTN, glycerol trinitrate-mediated vasodilatation; HC, healthy controls; PWV, pulse wave velocity.

Statistical analysis using Student's *t* test or Welch's test as appropriate.

Significant differences between the groups ($P < .05$) are shown in bold.

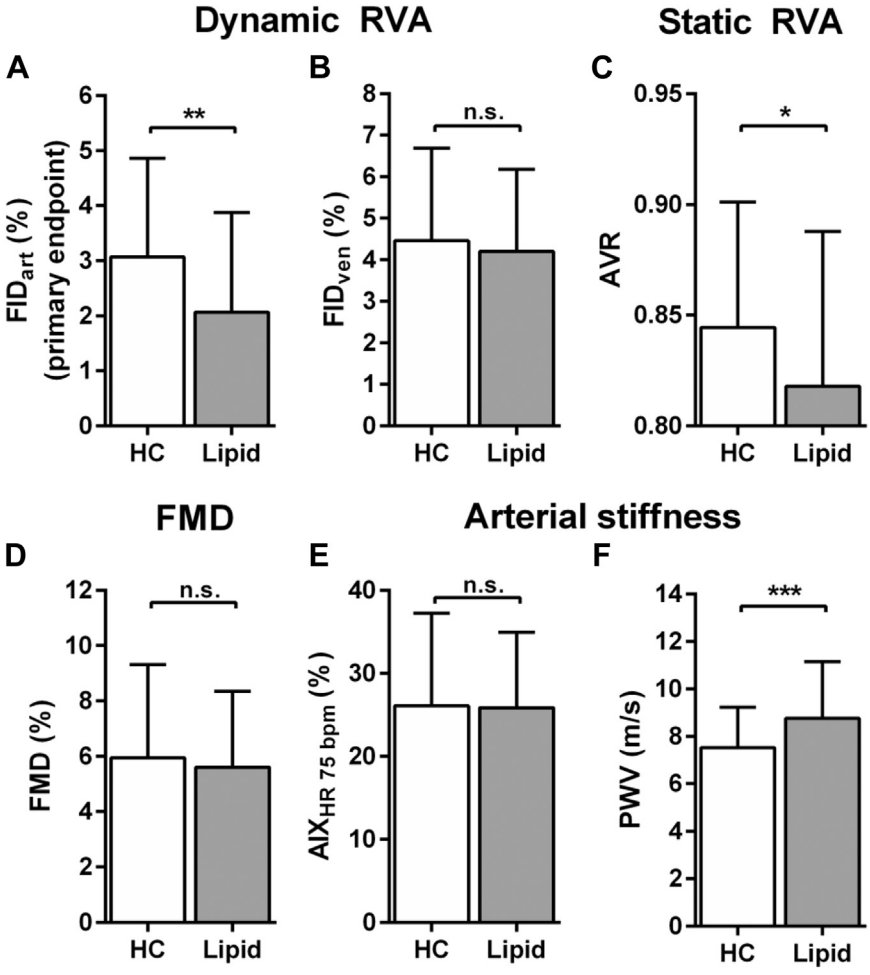

Figure 1 Vascular outcomes in patients with hypercholesterolemia (lipid group) and healthy controls (HC group). Dynamic RVA with assessment of the primary end point FID_{art} (A); measurement of secondary outcomes including FID_{ven} (B); static RVA with AVR (C); FMD (D); and markers of arterial stiffness, augmentation index, normalized to heart rate of 75 beats/min (AIX_{HR75}) (E) and PWV (F). The bars depict mean values ± standard deviation. ***$P < .001$, **$P < .01$, *$P < .05$. AVR, arteriovenous ratio; FID_{art}, flicker-induced dilatation of retinal arterioles; FID_{ven}, flicker-induced dilatation of retinal venules; FMD, flow-mediated vasodilatation; HC, healthy controls; PWV, pulse wave velocity; RVA, retinal vessel analysis.

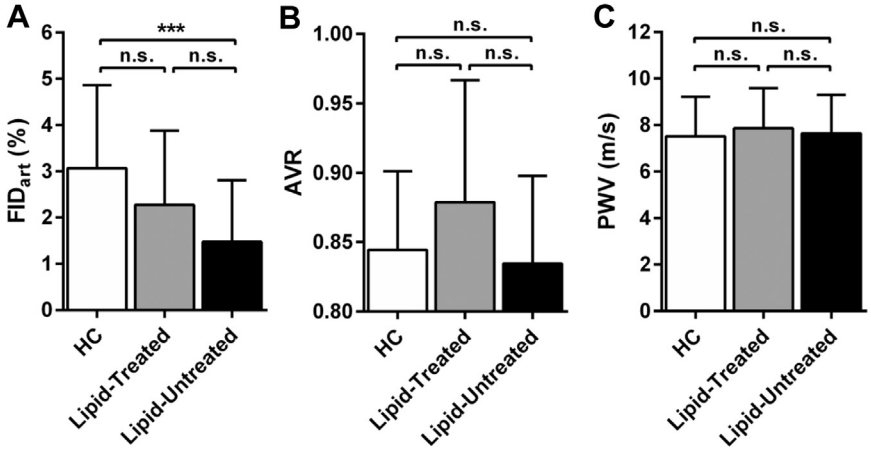

Figure 2 Vascular outcomes in subgroup of patients with hypercholesterolemia without hypertension and either treated with lipid-lowering drugs (lipid-treated, n = 8) or not on lipid-lowering therapy (lipid-untreated; n = 22) compared with healthy controls (HC; n = 78). FID_{art} (A), retinal AVR (B), and PWV (C) are shown. Statistical analysis was carried out using analysis of variance with Student's *t*-test post hoc. The bars depict mean values ± standard deviation. ***$P < .001$. AVR, arteriovenous ratio; FID_{art}, flicker-induced dilatation of retinal arterioles; HC, healthy controls; PWV, pulse wave velocity.

Table 3 Multiple linear regression of the primary end point FID_{art}

	FID_{art}		
Parameter	Estimate ± Std error	Standardized estimate (ß)	*P* value
Age	−0.03 ± 0.01	−0.19	**.03**
BMI	−0.03 ± 0.05	−0.05	.52
Systolic BP	0.02 ± 0.01	0.17	.06
Antihypertensive drugs	0.05 ± 0.29	0.02	.87
Lipid-lowering drugs	0.38 ± 0.27	0.17	.17
LDL cholesterol	−0.45 ± 0.16	−0.25	**.007**
HDL cholesterol	−0.05 ± 0.41	−0.01	.90
Overall regression	F ratio = 2.2, r^2 = 0.10, r^2 adj = 0.06, ***P* = .04**		

Adj, adjusted; BP, blood pressure; FID_{art}, flicker-induced dilatation of retinal arterioles; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Significant differences between the groups ($P < .05$) are shown in bold.

available. Reimann *et al.*[5] investigated flicker-induced retinal dilatation in 21 hypercholesterolemia patients before and after LDL apheresis. The authors noted a high frequency of impaired FID_{art} and FID_{ven} in patients with hypercholesterolemia compared with reference values (no control group was available). However, the study was performed with a significant proportion of patients with established cardiovascular disease, limiting comparability to our data. Interestingly, the authors noted improved FID_{ven} and a trend of improved FID_{art} after LDL apheresis, suggesting that retinal microvascular dysfunction due to hypercholesterolemia may be reversible. The plasticity of FID_{art} has also been recently shown in an interventional study using dietary fat loading, which resulted in acutely impaired retinal vasodilatation in response to flicker light.[20] While our study was not powered to assess differences between subjects with and without lipid-lowering therapy, FID_{art} appeared to be more impaired in untreated hypercholesterolemia patients. More studies are needed with longitudinal evaluation of FID_{art} in response to lipid-targeted interventions.

Unexpectedly, although there was a trend of lower FMD in hypercholesterolemia patients, we did not observe a significant difference between patients and controls. As our study was not powered for the secondary end points, it may have lacked adequate power to assess differences in FMD. The mild difference in LDL cholesterol of around 0.8 mmol/L (30 mg/dL) between the groups may also be a possible explanation. In addition, a previous study found only a weak correlation between FMD and FID_{art},[6] whereas in our study, no relevant correlation between the 2 parameters was shown. This may point toward important differences in the mechanism of vascular dilatation.

While shear stress–mediated NO release from endothelial cells is a major mechanism in FMD, the exact mechanism for retinal dilatation in response to flicker light is still unclear. Signaling from retinal neuronal cells to retinal blood vessels in response to the metabolic stress of flicker light appears to be the dominant mechanism (collectively termed "neurovascular coupling").[21] Neurovascular coupling involves potassium release from retinal neurons with direct vasodilatatory action, release of vasodilatatory metabolic byproducts such as lactate, adenosine, or carbon dioxide, neurotransmitter-mediated activation of retinal glial cells with release of prostaglandins that act on retinal vessels and production of NO by retinal neuronal cells with diffusion to blood vessels resulting in vasorelaxation.[21] The importance of NO signaling in mediating neurovascular coupling has been shown by a study in which the NO synthase inhibitor N-methylarginine (L-NMMA) reduced flicker-induced dilatation of both retinal arterioles and venules to the level of normal vessel pulsations in humans.[4]

Thus, in contrast to FMD, reduced flicker-induced dilatation may not only be a marker for vascular but also neuronal or glial cell dysfunction. Interestingly, hypercholesterolemia may affect retinal neuronal cell populations also via nonvascular mechanisms. In animal models, hypercholesterolemia increases the deposition of lipid byproducts in the Bruch's membrane and within retinal astrocytes and Müller cells, which promotes ischemia, oxidative stress, as well as cytotoxic glutamate signaling in retinal neuronal cells.[22] Accordingly, treatment with statins reduces extravascular lipid depositions and retinal neuronal cell death in these models.[23] Future studies will need to further evaluate the reproducibility of RVA compared with other methods and study the mechanisms underlying flicker-induced retinal vasodilatation. Animal models may be helpful for exploring these mechanisms. An adapted dynamic RVA method has previously been established in rats.[24]

While we found an initial difference in AVR and PWV between patients with hypercholesterolemia and controls, the difference disappeared when analysis was restricted to participants without hypertension. Likewise, systolic BP but not LDL cholesterol was significantly associated with AVR in the multivariable analysis. This supports earlier data that retinal arteriolar constriction represents more an indicator or consequence of hypertension, which is also supported by the close association of AVR with stroke risk.[25] A lower AVR in hypertensive patients may also explain the lack of association of FID_{art} with systolic BP as the prevasoconstricted state may allow more reserve for vasodilatation.[26]

Interestingly, AVR correlated negatively with PWV of the aorta, pointing toward a relationship of retinal microvascular constriction with arterial stiffening of larger conduit arteries, which is in line with a previous study.[27]

Limitations

Owing to the observational nature of the study, unmeasured confounding between patients with hypercholesterolemia and controls is possible. To reduce confounding and focus the analysis on the effect of hypercholesterolemia alone, smokers and patients with cardiovascular diseases, diabetes, or significant eye pathologies were excluded, as these conditions are known to affect retinal microvascular function.[3] Therefore we cannot extend our findings to patients with a combination of several risk factors (such as diabetes, hypercholesterolemia, and smoking), which is known to potentiate the risk of cardiovascular disease.[28] Despite our exclusion criteria, our patient sample was enriched with patients with coexisting hypertension, a condition known to be closely associated with hypercholesterolemia. As the association of hypercholesterolemia with retinal microvascular dysfunction remained in both normotensive dyslipidemic subjects and in the multivariable analysis, our results appear to be robust. The lack of difference in HDL cholesterol between both groups represents a limitation that may partly explain the lack of association of HDL cholesterol with FID_{art}. At last, our study was cross-sectional and did not study the temporal evolution of retinal microvascular dysfunction in hypercholesterolemia. Longitudinal and interventional studies are needed to further test the clinical value of this method.

Conclusion

This observational study on RVA in hypercholesterolemia found a significant degree of retinal microvascular dysfunction in patients with hypercholesterolemia, evidenced by a significant reduction in FID_{art}. LDL, but not HDL, cholesterol was a significant negative predictor of FID_{art}, highlighting the adverse effect of hypercholesterolemia on the retinal microcirculation. Dynamic RVA may be a promising method for the noninvasive study of microvascular endothelial dysfunction in populations at risk for cardiovascular disease.

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Appendix

Table S1 Multiple linear regression of other vascular parameters

Parameter	FID_{ven} Estimate ± Std error	FID_{ven} Standardized estimate (ß)	FID_{ven} *P*-value	AVR Estimate ± Std error	AVR Standardized estimate (ß)	AVR *P*-value
Age	−0.003 ± 0.02	−0.02	.85	−0.0004 ± 0.0005	−0.08	.35
BMI	−0.11 ± 0.06	−0.17	.08	−0.002 ± 0.002	−0.10	.25
Systolic BP	0.009 ± 0.01	0.06	.51	−0.001 ± 0.0004	−0.26	**.003**
Antihypertensive drugs	−0.15 ± 0.34	−0.05	.66	0.008 ± 0.01	0.09	.41
Lipid-lowering drugs	0.16 ± 0.32	0.06	.62	−0.0006 ± 0.009	−0.007	.95
LDL cholesterol	0.11 ± 0.19	0.05	.58	0.0001 ± 0.005	0.002	.98
HDL cholesterol	0.29 ± 0.48	0.06	.54	0.02 ± 0.01	0.16	.08
Overall regression	F ratio = 0.84, r^2 = 0.04, r^2 adj = −0.007, *P* = .55			F ratio = 4.0, r^2 = 0.17, r^2 adj = 0.13, *P* = **.0005**		
Parameter	CRAE Estimate ± Std error	CRAE Standardized estimate (ß)	CRAE *P*-value	CRVE Estimate ± Std error	CRVE Standardized estimate (ß)	CRVE *P*-value
Age	−0.25 ± 0.13	−0.17	**.0459**	−0.18 ± 0.14	−0.12	.18
BMI	−0.13 ± 0.45	−0.03	.77	0.37 ± 0.50	0.07	.45
Systolic BP	−0.36 ± 0.10	−0.31	**.0005**	−0.14 ± 0.11	−0.11	.20
Antihypertensive drugs	−0.84 ± 2.44	−0.04	.73	−3.34 ± 2.67	−0.15	.21
Lipid-lowering drugs	1.15 ± 2.29	0.06	.62	1.4 ± 2.51	0.07	.58
LDL cholesterol	−0.18 ± 1.38	−0.01	.89	−0.27 ± 1.51	−0.02	.86
HDL cholesterol	0.58 ± 3.45	0.02	.87	−5.82 ± 3.78	−0.15	.13
Overall regression	F ratio = 3.6, r^2 = 0.16, r^2 adj = 0.11, *P* = **.002**			F ratio = 1.6, r^2 = 0.07, r^2 adj = 0.03, *P* = .15		
Parameter	FMD Estimate ± Std error	FMD Standardized estimate (ß)	FMD *P*-value	GTN Estimate ± Std error	GTN Standardized estimate (ß)	GTN *P*-value
Age	−0.045 ± 0.03	−0.16	.08	−0.10 ± 0.05	−0.18	**.04**
BMI	−0.02 ± 0.09	−0.02	.81	−0.12 ± 0.17	−0.07	.46
Systolic BP	−0.005 ± 0.02	−0.02	.79	−0.03 ± 0.04	−0.06	.48
Antihypertensive drugs	0.09 ± 0.49	0.02	.85	2.2 ± 0.88	0.3	**.02**
Lipid-lowering drugs	0.10 ± 0.46	0.003	.98	−1.55 ± 0.83	−0.22	.07
LDL cholesterol	0.22 ± 0.28	0.07	.43	0.57 ± 0.51	0.1	.27
HDL cholesterol	1.1 ± 0.70	0.15	.31	0.69 ± 1.25	0.05	.58
Overall regression	F ratio = 1.1, r^2 = 0.05, r^2 adj = 0.003, *P* = .39			F ratio = 2.6, r^2 = 0.12, r^2 adj = 0.07, *P* = **.02**		
Parameter	PWV Estimate ± Std error	PWV Standardized estimate (ß)	PWV *P*-value	AIX_{HR75} Estimate ± Std error	AIX_{HR75} Standardized estimate (ß)	AIX_{HR75} *P*-value
Age	0.09 ± 0.13	0.43	**<.0001**	0.28 ± 0.07	0.29	**.0002**
BMI	0.02 ± 0.05	0.03	.71	−0.12 ± 0.27	−0.04	.65
Systolic BP	0.05 ± 0.01	0.32	**<.0001**	0.15 ± 0.06	0.20	**.01**
Antihypertensive drugs	−0.26 ± 0.26	−0.09	.33	0.52 ± 1.43	0.04	.71
Lipid-lowering drugs	−0.15 ± 0.24	−0.06	.54	1.51 ± 1.34	0.12	.26
LDL cholesterol	−0.002 ± 0.15	−0.0008	.99	0.29 ± 0.81	0.03	.72
HDL cholesterol	−0.40 ± 0.37	−0.08	.28	7.23 ± 2.03	0.31	**.0005**
Overall regression	F ratio = 15, r^2 = 0.44, r^2 adj = 0.41, ***P*** **< .0001**			F ratio = 6.7, r^2 = 0.2, r^2 adj = 0.22, ***P*** **< .0001**		

Adj, adjusted; AIX, augmentation index normalized to heart rate of 75 beats/min; AVR, arteriovenous ratio; BP, blood pressure; CRAE, central retinal artery equivalent; CRVE, central retinal vein equivalent; FID_{art}, flicker-induced dilatation of retinal arterioles; GTN, glycerol trinitrate-mediated vasodilatation; FMD, flow-mediated vasodilatation; HDL, high-density lipoprotein; LDL, low-density lipoprotein; PWV, pulse wave velocity.

Significant values (*P* < .05) are shown in bold.

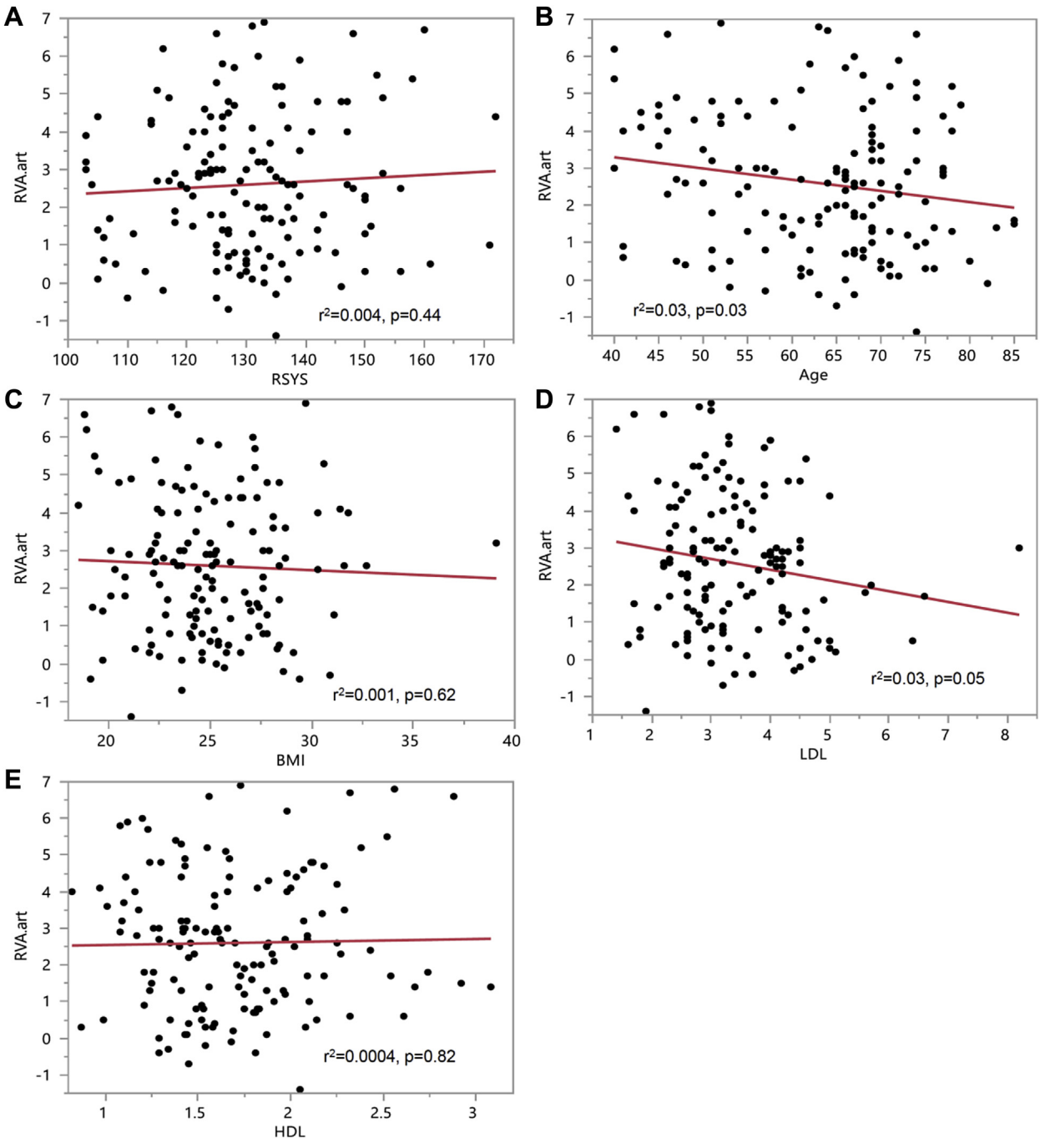

Figure S1 Univariate regression of flicker-induced dilatation of retinal arterioles (FID_{art}) with systolic blood pressure (A), age (B), body mass index (C), LDL cholesterol (D), and HDL cholesterol (E).