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Influence of remnant cholesterol on direct LDL and HDL cholesterol measurement accuracy

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Abstract

Objectives: Identifying the potential sources of bias in the direct measurement of low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) is important. In this study, we aimed to investigate the effect of remnant cholesterol on LDL-C and HDL-C levels measured using homogenous methods.

Methods: We obtained 41 commutable frozen serum samples and measured LDL-C and HDL-C levels. Eight measurement systems were used, and the degree of bias was obtained by comparing with the values obtained using the reference methods. Correlations among remnant cholesterol/LDL-C, remnant cholesterol/HDL-C, and bias were analyzed using Spearman's analysis.

Results: In all eight systems, samples with a positive bias >4 % had lower LDL-C levels and higher remnant cholesterol levels, as measured by the reference methods, compared to those with a bias ≤4 %. A significant correlation between remnant cholesterol/LDL-C and a positive bias of LDL-C was observed in six of the eight systems evaluated. For HDL-C bias, three systems showed a positive correlation, and three systems showed a negative correlation. In some systems, LDL-C bias was higher in samples with remnant cholesterol/LDL-C ≥0.25 than in those with remnant cholesterol/LDL-C <0.25.

Conclusions: Remnant cholesterol has a potential effect on direct LDL-C and HDL-C measurements, which has been observed when several measurement systems are used. For these systems, manufacturers should improve the methods to reduce the interference of remnant cholesterol.

Keywords: cardiovascular disease; high-density lipoprotein cholesterol; lipids; low-density lipoprotein cholesterol; remnant cholesterol

Introduction

Cardiovascular disease (CVD) is one of the leading causes of death worldwide [1]. Lipids and lipoproteins play pivotal roles in the development and progression of CVD [2]. Several large epidemiologic studies have reported the close relationship between these lipid and lipoprotein levels and the risk of CVD [3–6]. Among these, the opposite association with CVD risk of low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) has been extensively studied. LDL-C is well known for its association with the development of CVD [3–5]. By contrast, HDL-C has been shown to be associated with a decreased CVD risk [3, 6]. Owing to the importance of both cholesterols, their levels are used to identify patients who require treatment and monitor the efficacy of lipid-lowering therapy [7, 8]. Therefore, their accurate measurement has a pivotal role in the management of patients with CVD.

The reference methods for LDL-C and HDL-C measurement were proposed by the US Centers for Disease Control and Prevention (CDC). They comprise three steps, including ultracentrifugation, precipitation, and cholesterol measurement using the Abell–Kendall method [9, 10]. However, this method is time-consuming, laborious, and requires a large volume of samples, which limits its use in clinical laboratories [11]. Consequently, alternative methods for LDL-C and HDL-C measurement or estimation have been suggested. The Friedewald equation for LDL-C estimation has been developed [12] and is widely used owing to its simplicity and usefulness in routine and epidemiologic use

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[13]. However, the equation has well-known limitations: it cannot be applied to individuals with high levels of triglycerides (TG, >4.52 mmol/L) or low levels of LDL-C (<1.81 mmol/L), and fasting blood samples should be used for LDL-C estimation [14–16]. To overcome these limitations, improved LDL-C calculation equations, such as Martin–Hopkins [17] and Sampson [18], have been proposed and proven to be useful.

Direct homogeneous methods for measuring LDL-C and HDL-C levels have also been developed and are widely implicated in routine use [13]. These methods can be rapidly performed without preparation and are fully automated, resulting in improved precision and accuracy [11]. For accurate measurement, various reagents involve using methods that enable LDL-C or HDL-C to be selectively measured without interference from other components [13]. Direct measurement is a good alternative; however, the reliability of this method has been questioned for several reasons [19]. The measured value varies based on the reagent used [20, 21]. Lipids and lipoproteins can interfere with the directly measured values of LDL-C and HDL-C [22, 23]. The latter is a concern since some reagents also detect a part of cholesterol present in the chylomicron remnants, intermediate-density lipoprotein and very-low-density lipoprotein (VLDL), also known as remnant cholesterol [9, 24].

As accurate measurement of LDL-C and HDL-C is crucial in clinical management, the identification and elimination of potential sources, such as remnant cholesterol, that can cause bias in direct measurement is important. In this study, we aimed to investigate the effect of remnant cholesterol on LDL-C and HDL-C levels measured using direct methods.

Materials and methods

Samples

In this study, we used 41 commutable frozen serum samples that were produced following the Clinical and Laboratory Standards Institute (CLSI) C37-A guidelines from Severance Hospital, Konkuk University Medical Center, and Chungnam National University Hospital (Institutional Review Board approval numbers: 4-2017-0351 [Severance Hospital], 2022-03-037 [Konkuk University Medical Center], and 2022-09-078 [Chungnam National University Hospital]). Healthy donors who agreed to participate in this study were screened for eligibility according to standard blood donation criteria. For each eligible donor, 400 mL of whole blood was collected. The collection and subsequent processing were performed in accordance with CLSI C37-A guidelines to minimize sample alteration. Except for three samples (CFS 2931, CFS 2932, and CFS-22-03, which were pooled from 11, 8, and 12 donor units, respectively), all other samples were prepared by pooling blood from three to five donors overnight to create commutable frozen samples. No modifications or alterations were made during sample preparation. All prepared samples were stored at −70 °C until analysis. Informed consents were obtained from all donors. This study was conducted according to the guidelines for Good Clinical Practice and Declaration of Helsinki. These 41 samples were used in the Korean Quality Assurance Program for lipid assays between 2023 and 2024. Since 2011, the Korean Society of Laboratory Medicine has collaborated with the Korea Disease Control and Prevention Agency (KDCA) to implement the Laboratory Standardization Project (LSP) aimed at standardizing major tests including total cholesterol (TC), LDL-C, HDL-C, TG, creatinine, and HbA_{1c} levels. The Korean quality assurance program for manufacturers is a core component of the KDCA-LSP that is used to evaluate the quality of *in vitro* diagnostics. In this program, the KDCA provides two vials of each sample to the participating manufacturers. The manufacturers measure TC, LDL-C, HDL-C, and TG levels from each vial on two separate days, perform triplicate measurements, and submit the results to the KDCA. In 2023 and 2024, the Korean quality assurance program for lipid assay manufacturers was conducted twice a year, with 12 samples for each program. Overall, 48 samples were used over 2 years; however, seven samples were duplicated. For these duplicate samples, we included only the results from the first program in which they were used, resulting in an analysis of 41 independent sample results.

Reference value assignment

The reference values of TC, HDL-C, LDL-C, and TG in 41 samples were determined using reference methods. TC levels were quantified using the CDC-certified Abell–Kendall method at the KDCA. HDL-C and LDL-C levels were measured at the Canadian External Quality Assessment Laboratory (CEQAL in Vancouver, Canada). HDL-C levels were determined via the CDC ultracentrifugation using the heparin manganese precipitation method, while LDL-C concentrations were measured using the CDC beta-quantification technique. TG values were assigned based on measurements conducted at the KDCA using isotope dilution mass spectrometry. KDCA and CEQAL are highly specialized reference laboratories for lipid assays certified by the Cholesterol Reference Method Laboratory Network.

Furthermore, remnant cholesterol levels were calculated from the reference values of TC, HDL-C, and LDL-C using the following formula: remnant cholesterol=TC – HDL-C – LDL-C.

Bias estimation of measurement systems

Eight commercially available systems from Roche (Roche Diagnostics GmbH, Mannheim, Germany), Beckman Coulter (Beckman-Coulter, Brea, CA, USA), Siemens (Siemens Healthineers, Forchheim, Germany), Abbott (Abbott Laboratories, Abbott Park, IL, USA), FUJIFILM Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan), Minaris (Minaris Medical, Tokyo, Japan), SEKISUI (Sekisui Medical, Tokyo, Japan), and Diasys (DiaSys Diagnostic Systems GmbH, Holzheim, Germany) were used in the Korean quality assurance program conducted for manufacturers of lipid assays during 2023 and 2024. These systems were labeled A, B, C, D, E, F, G, and H, respectively. Details of the reagents, calibrators, and instruments used are provided in Supplementary Table S1. These systems were used to measure TC, HDL-C, LDL-C, and TG levels in 41 samples six times for each sample (two separate days in triplicate). The average of six measurements was used to estimate the measurement bias from the reference value. The measurement biases (%) of LDL-C and HDL-C were calculated for each sample in the eight systems. Additionally, lipoprotein(a) was measured using Roche Cobas 8000 platform.

Assessment classification of LDL-C direct methods and LDL-C estimation equations

We assessed the ability of eight direct LDL-C measurement systems to correctly classify samples based on the current guidelines [8], in which LDL-C levels of 1.42, 1.81, 2.59, 3.00, and 4.92 mmol/L are used as medical decision cut-off points. Each sample was classified based on the LDL-C value measured using the reference method, which was the standard for comparison. Similarly, the concordance rates of classifications based on LDL-C values calculated using three estimation formulas – Friedewald [12], Martin–Hopkins [17], and Sampson [18] – were also evaluated. In the Friedewald equation, a fixed TG/remnant cholesterol ratio of 2.2 for LDL-C calculation, expressed as: LDL-C (mmol/L)=TC – HDL-C – TG/2.2, is used. Meanwhile, for the Martin–Hopkins equation, TG/remnant cholesterol ratio was determined based on the strata of TG and non-HDL-C, and the value of LDL-C was calculated as: LDL-C (mmol/L)=TC – HDL-C – TG/(ratio determined based on TG and non-HDL-C). Using the Sampson equation, LDL-C was calculated as follows: LDL-C (mg/dL)=TC/0.948 – HDL-C/0.971 – (TG/8.56 + TG × Non-HDL-C/2140 – TG^2/16,100) – 9.44. Then calculated LDL-C values were converted to mmol/L by dividing by 38.67. For the LDL-C calculations for each system, the TC, HDL-C, and TG values measured in each system were used.

Statistical analyses

Continuous variables were compared using either the Student's t-test or the Wilcoxon rank-sum test, whereas Fisher's exact test was employed to compare concordance rates. The relationships between the remnant cholesterol/LDL-C or HDL-C ratio and direct LDL-C or HDL-C measurements were assessed using Spearman's correlation analysis. The same analysis was used for assessing the relationship between the TG/LDL-C or HDL-C ratio and direct LDL-C or HDL-C measurements. All statistical analyses were conducted using Analyse-it software (Analyse-it Software, Ltd., Leeds, UK) and R 4.3.2.

Results

Reference value assignment

The assigned reference values for the TC, HDL-C, LDL-C, and TG levels are presented in Supplementary Table S2. The mean TC value was 4.74 mmol/L (range, 3.79–6.35 mmol/L). The mean HDL-C value was 1.40 mmol/L (range: 0.90–1.87 mmol/L), and the mean LDL-C value was 2.76 mmol/L (range: 2.07–4.17 mmol/L). The mean TG value was 1.19 mmol/L (range: 0.66–2.64 mmol/L). The mean calculated remnant cholesterol was 0.58 mmol/L, with a range of 0.24–1.42 mmol/L. A significant correlation was observed between TG and remnant cholesterol levels ($r=0.945$, $p<0.001$).

LDL-C, TG, remnant cholesterol and direct LDL-C measurement bias

The LDL-C measurement bias varied across samples and measurement systems. In the following analysis, only positive bias values (measured LDL-C values higher than the reference) were considered.

The number of samples exceeding a positive bias of 4 % varied considerably, from as few as one to as many as 20, depending on the measurement system. When comparing mean LDL-C, TG and remnant cholesterol levels (all measured by reference methods) between samples with bias >4 % and those with bias ≤4 %, samples with bias >4 % showed

Table 1: Mean LDL-C and remnant cholesterol levels according to bias percentage (>4 % vs. ≤4 %) in each direct LDL-C measurement system.

Measurement system	Sample number, Positive bias >4 %	Mean LDL-C, mmol/L[a]			Mean TG, mmol/L[a]			Mean remnant cholesterol, mmol/L[a]		
		Positive bias >4 %	Positive bias ≤4 %	p-Value	Positive bias >4 %	Positive bias ≤4 %	p-Value	Positive bias >4 %	Positive bias ≤4 %	p-Value
A	1	2.27	2.77	0.293	1.24	1.18	0.642	1.06	0.57	0.163
B	10	2.50	2.84	0.049	1.78	0.99	0.002	0.91	0.48	<0.001
C	9	2.53	2.83	0.137	1.68	1.05	0.001	0.83	0.51	0.002
D	20	2.68	2.83	0.349	1.39	0.98	0.005	0.73	0.44	0.001
E	19	2.58	2.91	0.051	1.43	0.97	0.004	0.70	0.48	0.012
F	2	2.18	2.79	0.039	1.85	1.15	0.138	1.24	0.55	0.027
G	7	2.40	2.83	0.010	1.82	1.05	0.001	0.88	0.52	0.007
H	2	2.18	2.79	0.039	1.85	1.15	0.138	1.24	0.55	0.027

[a]Values measured by reference methods. LDL-C, low-density lipoprotein cholesterol; TG, triglycerides.

significantly lower LDL-C (system B, F, G, and H) and higher TG (system B, C, D, E, and G) and remnant cholesterol levels (all systems except A) (Table 1).

Correlation between the TG/LDL-C ratio and direct LDL-C measurement bias

The ratio of TG to LDL-C was calculated for each sample using the reference values of LDL-C and TG (Table 2). The mean TG/LDL-C ratio was 0.44, with the individual ratios ranging from 0.21–1.17.

We examined whether the TG/LDL-C ratio is associated with LDL-C bias in each measurement system and observed positive correlations in five of the eight systems (Figure 1B–E, G). These results suggest that when samples contain more TG relative to LDL-C, the LDL-C measurements of some direct methods may be falsely elevated. Because TG levels are closely related to remnant cholesterol levels, elevated remnant cholesterol in high-TG samples might be the main cause of LDL-C bias in such cases.

Correlation between the remnant cholesterol/LDL-C ratio and direct LDL-C measurement bias

To examine whether the remnant cholesterol/LDL-C ratio influences the accuracy of direct LDL-C measurements, the ratio of remnant cholesterol to LDL-C was calculated for each sample using the reference values of LDL-C and remnant cholesterol (Table 2). The mean remnant cholesterol/LDL-C ratio was 0.22, with the individual ratios ranging from 0.08 to 0.68.

Correlation analyses were performed to assess the relationship between the remnant cholesterol/LDL-C ratio and direct LDL-C measurement bias (Figure 2). Statistically significant positive correlations were observed in six of the eight systems (Figure 2B–E, G, and H). These results suggest that when there is more remnant cholesterol relative to LDL-C in samples, the LDL-C measurement of some direct methods may be falsely elevated, and this positive bias increases with the remnant cholesterol/LDL-C ratio. Using the Deming regression analysis, we determined the threshold of the remnant cholesterol/LDL-C ratio at which the positive bias exceeded 4 %. The affected measurement systems, systems B–E, G, and H, showed threshold values ranging from 0.22 to 0.33, which varied by manufacturer (Supplementary Table S3). To investigate the influence of lipoprotein(a) on LDL-C bias, the correlation between lipoprotein(a) and LDL-C bias was also assessed. No association was observed in any of the eight measurement systems (Supplementary Table S4).

Correlation between the TG/HDL-C ratio or remnant cholesterol/HDL-C ratio and direct HDL-C measurement bias

The associations of TG and remnant cholesterol with direct HDL-C measurement bias were also investigated (Table 3). The mean TG/HDL-C ratio was 0.91 (range, 0.39–2.48), and the mean remnant cholesterol/HDL-C ratio was 0.45 (range, 0.15–1.31).

Table 2: Remnant cholesterol/LDL cholesterol ratio, triglyceride/LDL cholesterol ratio, and % bias from LDL cholesterol reference value for each direct LDL cholesterol measurement system.

Commutable frozen samples	Remnant cholesterol/LDL-C	Triglycerides/LDL-C	% Bias from LDL-C reference value in each measurement system							
			A	B	C	D	E	F	G	H
CFS-16-YA	0.11	0.27	1.9	1.4	−1.2	−1.4	3.2	0.0	−1.0	−2.7
CFS-19-YA	0.13	0.31	0.5	−0.1	−4.4	−0.9	0.5	−1.6	−2.6	−4.2
CFS-19-YC	0.15	0.31	2.2	0.9	−1.7	0.3	3.6	1.9	−2.3	−0.3
CFS-20-YA	0.18	0.39	1.6	3.6	0.3	0.8	7.8	−0.9	0.2	−3.2
CFS-20-YC	0.14	0.25	2.4	1.0	−1.8	2.0	−0.8	1.7	−1.5	−0.4
CFS-21-YC	0.09	0.22	0.3	−2.9	−6.6	−1.8	−3.1	−0.4	−1.2	−3.1
CFS-2018-LOW	0.40	0.76	3.2	16.7	10.8	5.3	11.8	1.4	−7.8	1.3
CFS-16-07	0.27	0.58	3.7	8.9	3.1	6.5	10.4	2.0	4.3	−1.9
CFS-16-08	0.27	0.53	−1.3	4.5	−1.1	2.8	5.2	−1.7	0.1	−3.1
CFS 2734	0.11	0.25	1.2	−4.0	−7.9	−3.1	−5.3	0.1	−8.9	−3.0
CFS-CNU-1901	0.16	0.35	2.5	3.0	−0.9	−0.3	5.9	0.2	−0.7	−2.4
CFS-CNU-2001	0.18	0.36	3.6	6.3	1.1	3.0	4.8	1.9	2.1	0.7
CFS-CNU-1801	0.16	0.28	−4.7	0.9	4.7	4.1	5.5	1.5	0.5	0.1
CFS 2931	0.15	0.39	−11.5	−7.4	0.1	−0.5	−0.9	−3.8	−4.4	−4.1
CFS-18-YB	0.24	0.49	−4.3	0.9	7.2	5.7	3.9	0.5	2.8	0.2
CFS-22-YA	0.16	0.36	−10.3	−3.0	1.1	1.0	0.0	−4.5	−1.3	−6.8
CFS-22-03	0.14	0.36	−6.6	0.6	3.2	−0.3	4.1	−2.9	1.7	−4.9
CFS-22-YB	0.14	0.28	−4.1	0.0	3.2	2.9	1.8	−0.1	0.4	−2.1
CFS-19-YB	0.13	0.31	−6.6	−3.5	−0.5	2.0	0.8	−1.9	−2.7	−1.8
CFS-CNU-1804	0.68	1.17	−2.0	14.8	19.8	10.9	20.4	7.4	8.7	11.5
CFS 2932	0.24	0.58	−7.9	2.6	6.6	1.0	8.8	−1.7	3.6	−3.8
CFS2735	0.35	0.85	−11.8	6.3	8.2	3.2	10.9	−3.9	6.2	−5.3
CFS-2018-MID	0.38	0.78	−4.7	9.1	12.1	6.8	14.8	1.0	9.8	−0.6
CFS-22-YC	0.18	0.29	0.2	−2.5	2.4	5.1	0.3	3.9	−0.2	2.5
CFS-18-YA	0.19	0.43	−2.2	0.8	2.8	9.1	4.1	−1.0	0.6	0.6
CFS-20-YB	0.15	0.30	−0.8	−2.1	1.2	6.9	0.2	0.1	−0.7	0.8
CFS-21-YA	0.14	0.34	−4.3	−4.6	−3.2	4.4	0.2	−3.4	−2.7	−2.1
CFS-16-09	0.12	0.26	−1.6	−6.9	−5.5	2.5	4.8	−1.2	−4.1	0.4
CFS-CNU-1802	0.23	0.44	−1.6	3.1	6.1	13.3	7.0	0.7	3.7	2.8
CFS-CNU-1805	0.18	0.40	−3.4	3.1	3.0	10.8	8.7	−1.4	4.1	−0.6
CFS-CNU-1902	0.19	0.47	−3.4	−3.1	0.3	7.7	0.3	−0.6	−0.3	−0.2
CFS-21-YB	0.20	0.37	2.7	3.1	−2.4	7.3	0.9	−3.0	0.7	−1.0
CFS-23-YA	0.16	0.37	3.6	0.2	−3.9	5.0	−2.0	−2.7	−2.3	−1.3
CFS-23-YB	0.08	0.21	−0.5	−3.4	−8.7	0.3	−1.1	−6.3	−7.0	−7.5
CFS-23-YC	0.14	0.35	−1.5	−2.5	−8.5	1.3	−4.4	−7.6	−6.2	−6.9
CFS 2041	0.37	0.59	1.0	7.5	0.3	11.0	11.1	−4.0	−0.7	−0.7
CFS 2042	0.25	0.44	2.2	2.2	−4.5	6.6	3.9	−2.4	−5.7	−3.2
CFS 2043	0.47	0.55	6.8	−0.5	−2.3	8.9	4.0	4.8	−9.5	11.6
CFS 2044	0.22	0.39	−0.4	−7.9	−9.9	0.1	−6.6	−4.7	−10.6	−0.1
CFS-23-03	0.39	0.76	3.6	9.0	2.4	12.9	7.1	−1.7	5.6	0.5
CFS-CNU-1905	0.34	0.81	1.1	9.4	4.3	13.8	9.9	−2.8	5.4	0.7

LDL-C, low-density lipoprotein cholesterol.

The correlation between TG/HDL-C ratio and HDL-C bias was investigated. Unlike LDL-C, the correlations varied across measurement systems: positive correlations were observed in three of the eight systems (Figure 3B–D), whereas negative correlations were found in another three (Figure 3A, F, H). Similar associations were observed between the remnant cholesterol/HDL-C ratio and direct HDL-C measurement bias (Figure 4).

Comparison of bias and classification concordance between direct LDL-C measurement and estimation equations

For each system, the LDL-C levels obtained using direct measurement methods and three different calculation equations (Friedewald, Martin–Hopkins, and Sampson) were compared with those measured using the reference

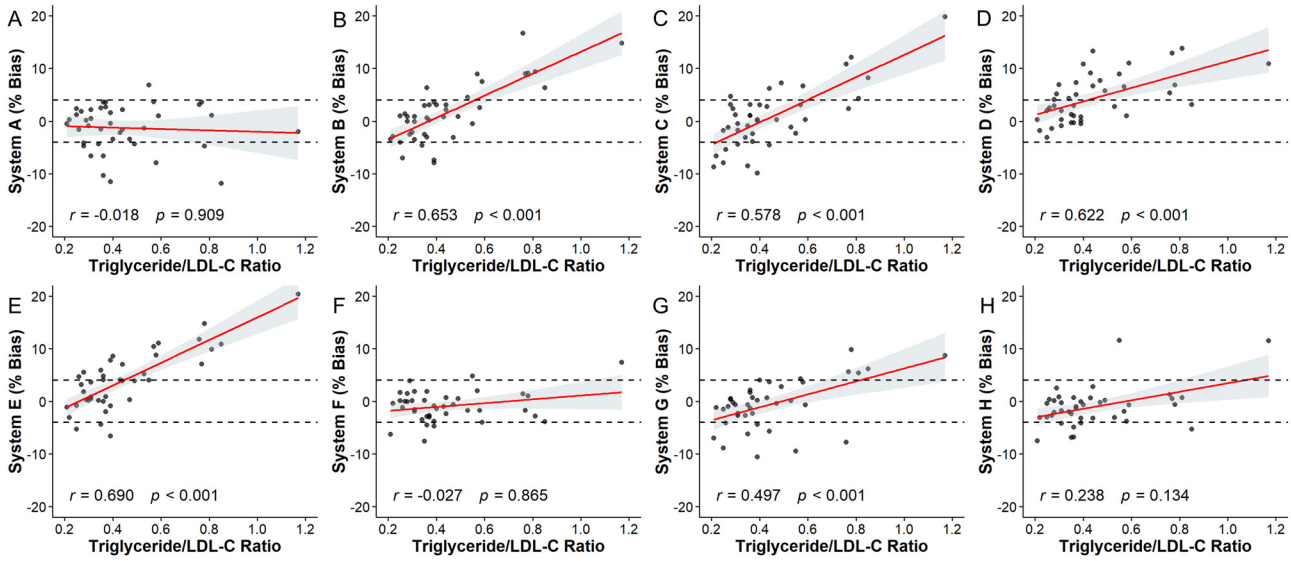

Figure 1: Spearman's correlation analysis between the TG/LDL cholesterol ratio and direct LDL-C %bias of each measurement system.

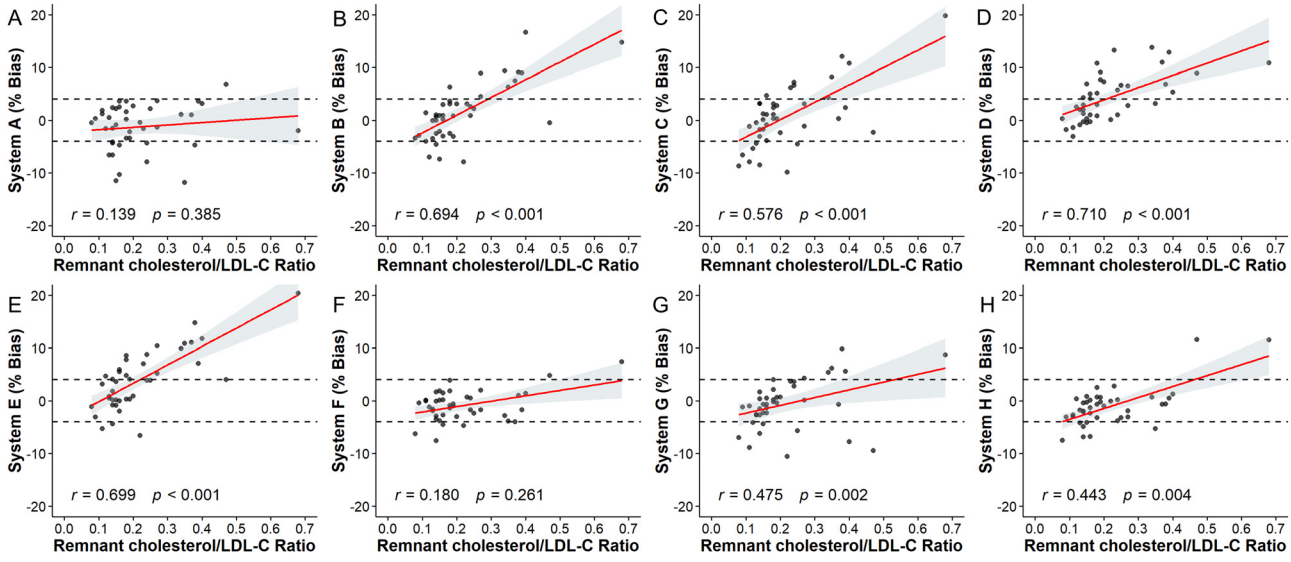

Figure 2: Spearman's correlation analysis between the remnant cholesterol/LDL cholesterol ratio and direct LDL-C %bias of each measurement system.

method. The bias and concordance at the cutoff of 1.42, 1.81, 2.59, 3.00, and 4.92 mmol/L based on reference LDL-C levels were analyzed (Table 4).

The mean bias of directly measured LDL-C was <4 % except for those measured using system D. The mean bias of all 41 samples for LDL-C ranged from −1.3 to 4.3 % based on the measurement system. Systems C and F exhibited a notably high degree of bias in calculated LDL-C, whereas the bias of LDL-C measured using the direct method was <4 %. For 31 samples with a low remnant cholesterol/LDL-C ratio (<0.25), direct LDL-C measurement using all the systems met the bias goal of ≤4 %. However, bias >4 % was still observed from calculated LDL-C in systems C and F. For 10 samples with a relatively high remnant cholesterol/LDL-C ratio (≥0.25), we observed a high mean bias of directly measured LDL-C (>4 %) in four measurement systems (System B, C, D and E). The calculated LDL-C in these samples also showed a high degree of bias, the extent of which varied by equation in most systems.

LDL-C determined using the eight direct assays correctly classified 82.9–95.1 % of samples. In six of the eight systems, the direct method had a higher correct classification rate than that of the estimation equations. Among the equations, the

Table 3: Remnant cholesterol/HDL cholesterol ratio, triglyceride/HDL cholesterol ratio, and % bias from HDL cholesterol reference value for each direct HDL cholesterol measurement system.

Commutable frozen samples	Remnant cholesterol/HDL-C	Triglycerides/HDL-C	% Bias from HDL-C reference value in each measurement system							
			A	B	C	D	E	F	G	H
CFS-16-YA	0.26	0.60	−0.7	3.4	4.8	−5.6	−2.0	2.5	1.4	0.0
CFS-19-YA	0.22	0.55	−0.6	1.7	1.8	−9.5	−3.7	3.6	0.3	1.4
CFS-19-YC	0.42	0.86	−2.2	1.6	2.7	−7.9	−4.8	1.3	0.6	0.6
CFS-20-YA	0.28	0.60	−0.9	1.0	0.9	−9.9	−4.5	2.9	−0.2	0.2
CFS-20-YC	0.36	0.66	−2.1	0.0	0.6	−10.3	−6.0	2.1	−1.4	−1.6
CFS-21-YC	0.17	0.44	0.9	−0.8	−0.4	−5.7	−4.6	4.6	−1.3	3.3
CFS-2018-LOW	1.31	2.48	−10.9	6.5	7.8	−2.9	−4.1	−5.8	5.8	−16.8
CFS-16-07	0.55	1.17	−2.8	2.9	4.3	−6.2	−3.2	1.8	0.6	−5.3
CFS-16-08	0.50	0.99	−0.9	5.2	6.1	−8.4	−2.2	3.2	2.7	−3.0
CFS 2734	0.18	0.42	−1.1	1.4	0.0	−9.9	−3.8	1.8	−1.0	−1.4
CFS-CNU-1901	0.36	0.81	−3.1	1.0	3.0	−8.5	−4.8	0.6	−0.7	−4.5
CFS-CNU-2001	0.29	0.57	−0.9	1.7	3.3	−8.7	−6.3	2.7	−0.3	−2.1
CFS-CNU-1801	0.24	0.41	−3.0	6.7	6.4	1.8	−6.7	0.7	2.5	−4.3
CFS 2931	0.23	0.59	−4.4	4.9	2.9	−0.6	−5.2	0.6	−0.6	−4.5
CFS-18-YB	0.45	0.92	−3.1	4.5	0.5	−0.6	−7.1	1.1	−0.7	−1.8
CFS-22-YA	0.29	0.65	−1.0	6.0	4.2	2.2	−3.3	3.5	0.4	−0.5
CFS-22-03	0.42	1.06	−6.6	6.0	2.9	2.9	−4.9	−1.9	0.6	−6.3
CFS-22-YB	0.31	0.60	−1.9	4.5	3.4	0.0	−5.7	2.7	0.7	−3.3
CFS-19-YB	0.23	0.53	−0.8	4.0	4.3	−0.7	−5.1	2.9	−0.2	−1.2
CFS-CNU-1804	1.27	2.18	−5.8	13.7	10.6	6.8	−5.4	0.3	6.3	−3.2
CFS 2932	0.51	1.20	−8.0	6.4	5.3	1.5	−6.3	−3.5	1.9	−7.1
CFS2735	0.60	1.46	−8.9	7.5	5.6	2.0	−5.0	−4.0	1.1	−10.2
CFS-2018-MID	0.70	1.44	−9.1	6.5	3.9	2.1	−3.9	−4.1	0.3	−9.1
CFS-22-YC	0.36	0.58	1.5	4.0	6.7	−0.4	−5.1	4.4	0.5	1.4
CFS-18-YA	0.33	0.74	−4.8	1.7	1.7	−0.4	−6.3	−1.0	1.3	−7.2
CFS-20-YB	0.31	0.63	−2.8	2.5	0.6	−0.8	−8.2	0.8	1.2	−5.1
CFS-21-YA	0.26	0.60	−2.5	4.3	4.9	1.1	−4.7	1.9	1.9	−9.1
CFS-16-09	0.17	0.39	1.2	4.1	4.5	0.9	−8.0	0.9	2.0	−9.2
CFS-CNU-1802	0.59	1.11	−7.9	9.9	8.1	6.9	−7.3	−2.4	1.5	−2.1
CFS-CNU-1805	0.30	0.67	−7.5	7.5	4.8	2.7	−7.0	−2.7	3.1	−9.9
CFS-CNU-1902	0.32	0.79	−5.1	2.9	2.2	−1.2	−8.5	−1.5	0.5	−8.3
CFS-21-YB	0.39	0.73	2.1	6.3	6.4	1.2	−2.0	4.9	4.5	−0.2
CFS-23-YA	0.26	0.60	−1.5	1.2	1.0	−4.4	−7.8	−0.5	−1.2	−3.7
CFS-23-YB	0.15	0.42	0.6	3.5	2.6	−2.1	−6.0	2.7	14.8	0.4
CFS-23-YC	0.22	0.58	1.8	3.3	1.8	−2.0	−3.6	3.7	0.5	−2.0
CFS 2041	1.05	1.68	−8.5	19.7	14.4	13.4	−2.7	−3.8	9.6	−13.3
CFS 2042	0.63	1.11	−9.0	13.3	10.0	7.5	−5.3	−5.0	4.9	−14.8
CFS 2043	0.82	0.97	−3.3	11.5	8.7	5.0	−7.4	−1.0	−2.1	−9.4
CFS 2044	0.34	0.60	−2.3	6.4	5.0	−1.0	−8.2	−1.4	2.4	−10.9
CFS-23-03	1.20	2.33	−6.4	10.3	7.3	6.1	−7.3	−3.3	4.4	−9.9
CFS-CNU-1905	0.75	1.77	−9.4	3.6	1.2	−0.9	−6.8	−5.4	−0.8	−9.0

HDL-C, high-density lipoprotein cholesterol.

Friedewald equation showed better concordance (87.8 %) than direct measurement (85.4 %) in system A. In system H, all three equations showed better concordance (87.8–90.2 %) than direct measurement (85.4 %). For samples with a low remnant cholesterol/LDL-C ratio (<0.25), the direct method had a higher correct classification rate than the estimation equations, except in system A. Similarly, for samples with a relatively high remnant cholesterol/LDL-C ratio (≥0.25) the direct method had a higher correct classification rate than the estimation equations, except in system G. The optimal

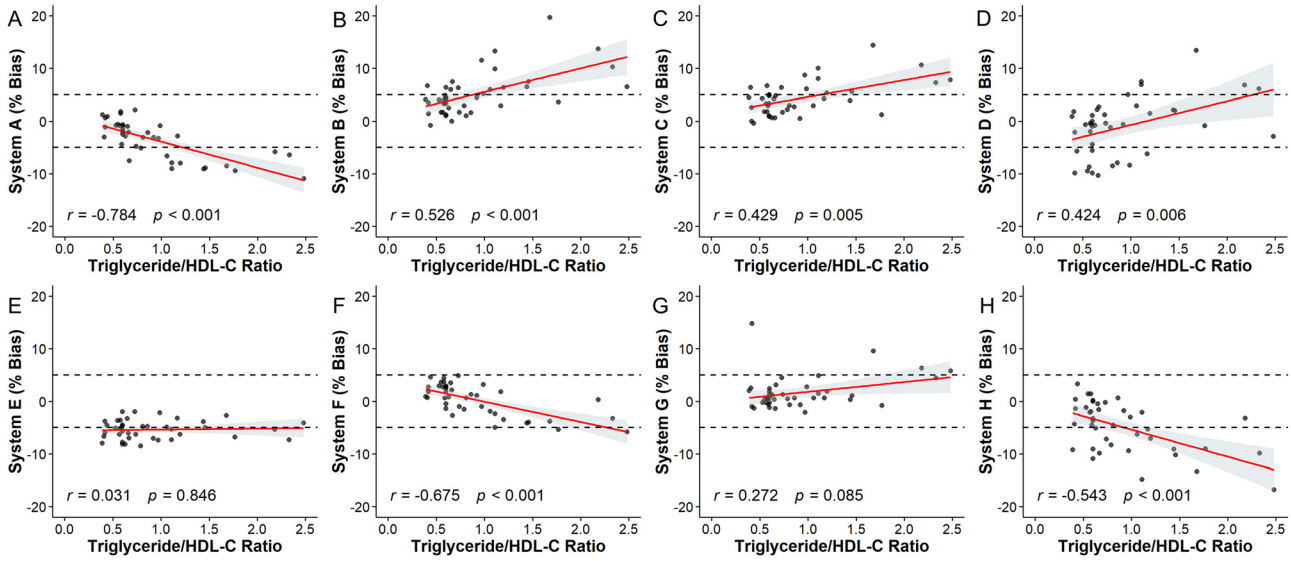

Figure 3: Spearman's correlation analysis between the TG/HDL cholesterol ratio and direct HDL-C %bias of each measurement system.

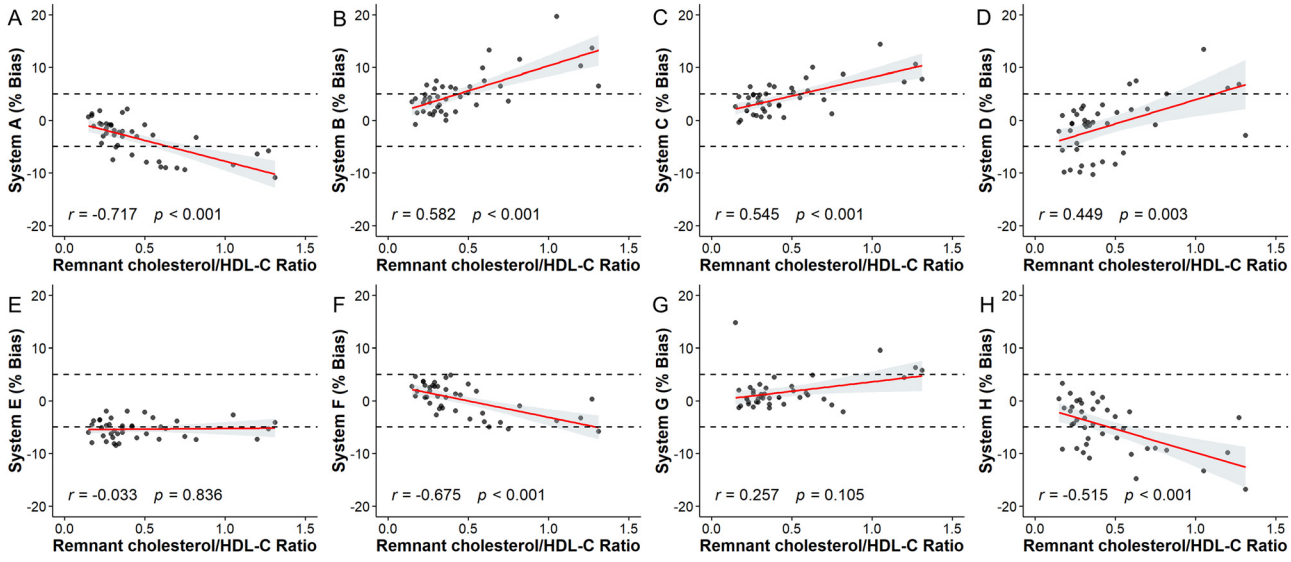

Figure 4: Spearman's correlation analysis between the remnant cholesterol/HDL cholesterol ratio and direct HDL-C %bias of each measurement system.

estimation equation with the highest LDL-C classification agreement varied, depending on the system used.

Discussion

In this study, we investigated the effect of remnant cholesterol on the bias in LDL-C and HDL-C levels measured using direct homogeneous methods. The results showed that a higher remnant cholesterol/LDL-C ratio was significantly associated with a positive bias in direct LDL-C measurement for most tested reagents. For HDL-C, a higher remnant cholesterol/HDL-C ratio was also significantly associated with bias; nevertheless, the effect varied among the reagents. These findings suggest that the presence of elevated remnant cholesterol could partly explain the discrepancies in the measured levels of LDL-C and HDL-C observed between the direct homogenous and reference methods.

The principle of homogenous assays is to selectively measure LDL-C without interference from other lipoproteins; however, several studies show that cholesterol from chylomicron and VLDL may be partially detected in these

Table 4: Mean bias and appropriate classification of LDL-C by direct methods and the equations with beta quantification.

Measurement system	All (n=41)		Mean bias, %			Correctly classified, %[a]		
	Mean bias, %	Correctly classified, %[a]	Remnant cholesterol/LDL-C <0.25 (n=31)	Remnant cholesterol/LDL-C ≥0.25 (n=10)	p-Value	Remnant cholesterol/LDL-C <0.25 (n=31)	Remnant cholesterol/LDL-C ≥0.25 (n=10)	p-Value
System A								
Direct method	−1.3	85.4	−1.6	0.0	0.386	80.6	100	0.106
Friedewald equation	2.0	87.8	−0.1	8.3	<0.001	87.1	90.0	0.343
Martin–Hopkins	2.5	82.9	−0.9	13.2	<0.001	90.3	60.0	0.020
Sampson	3.8	85.4	1.1	12.0	<0.001	87.1	80.0	0.189
System B								
Direct method	1.6	95.1	−0.7	8.6	<0.001	93.5	100	0.311
Friedewald equation	−0.4	85.4	−1.1	1.6	0.285	83.9	90.0	0.385
Martin–Hopkins	0.3	85.4	−1.9	7.2	0.005	83.9	90.0	0.385
Sampson	1.6	85.4	0.2	5.8	0.039	83.9	90.0	0.385
System C								
Direct method	0.7	87.8	−0.9	5.8	0.016	83.9	100	0.091
Friedewald equation	−6.4	75.6	−7.0	−4.7	0.393	74.2	80.0	0.334
Martin–Hopkins	−5.5	80.5	−7.6	1.2	0.005	74.2	100	0.059
Sampson	−4.5	82.9	−5.8	−0.6	0.018	77.4	100	0.050
System D								
Direct method	4.3	82.9	3.1	8.2	0.003	80.6	90.0	0.291
Friedewald equation	3.3	78.0	2.7	5.3	0.259	74.2	90.0	0.169
Martin–Hopkins	3.9	73.2	1.7	10.6	<0.001	74.2	70.0	0.437
Sampson	5.2	70.7	3.9	9.3	0.009	67.7	80.0	0.276
System E								
Direct method	4.0	92.7	1.8	10.6	<0.001	90.3	100	0.159
Friedewald equation	2.6	80.5	1.7	5.3	0.218	77.4	90.0	0.302
Martin–Hopkins	2.5	80.5	0.1	9.9	0.007	80.6	80.0	0.393
Sampson	4.1	80.5	2.6	9.0	0.039	77.4	90.0	0.302
System F								
Direct method	−0.9	90.2	−1.3	0.3	0.323	90.3	90.0	0.300
Friedewald equation	7.9	63.4	6.4	12.5	0.028	58.1	80.0	0.120
Martin–Hopkins	7.8	58.5	4.8	17.1	<0.001	61.3	50.0	0.298
Sampson	9.6	58.5	7.5	16.1	<0.001	58.1	60.0	0.373
System G								
Direct method	−0.6	85.4	−1.5	2.2	0.121	87.1	80.0	0.189
Friedewald equation	0.4	87.8	−0.3	2.3	0.202	83.9	100	0.091
Martin-Hopkins	0.5	90.2	−1.7	7.3	<0.001	87.1	100	0.184
Sampson	2.0	87.8	0.7	6.2	0.002	83.9	100	0.091
System H								
Direct method	−1.1	82.9	−1.9	1.4	0.119	80.6	90.0	0.291
Friedewald equation	2.5	68.3	0.5	8.7	0.051	64.5	80.0	0.213

Table 4: (continued)

Measurement system	All (n=41)		Mean bias, %			Correctly classified, %[a]		
	Mean bias, %	Correctly classified, %[a]	Remnant cholesterol/LDL-C <0.25 (n=31)	Remnant cholesterol/LDL-C ≥0.25 (n=10)	p-Value	Remnant cholesterol/LDL-C <0.25 (n=31)	Remnant cholesterol/LDL-C ≥0.25 (n=10)	p-Value
Martin–Hopkins	2.4	68.3	−0.9	12.6	<0.001	64.5	80.0	0.213
Sampson	4.0	65.9	1.5	11.9	0.020	64.5	70.0	0.366

[a]Number of samples correctly classified/total number of samples at the cutoffs 1.42, 1.81, 2.59, 3.00, and 4.92 mmol/L. LDL, low-density lipoprotein cholesterol.

assays [13, 24–26]. For each LDL-C measurement reagent, a specific LDL-C measurement method exists, and it is well recognized that the recovery of VLDL, intermediate-density cholesterol, and even LDL-C can vary depending on the reagent [13]. Reportedly, recovery can vary with density depending on the reagent used, even within the VLDL and LDL fractions [25]. Our findings support this observation, revealing that the degree of bias correlates with elevated remnant cholesterol levels. It is well known that elevated TG levels can introduce bias in direct LDL-C quantification, with some reagents showing a clear positive bias [27]. Circulating TG levels are closely associated with remnant cholesterol. Furthermore, our findings show that the remnant cholesterol/LDL-C and the TG/LDL-C ratios exhibit similar correlations with direct LDL-C measurement bias; hence, it is plausible that remnant cholesterol, which tends to be elevated in specimens with high TG levels, mainly contributes to this bias.

The interference caused by remnant cholesterol suggested that it has been impossible to completely exclude interfering substances, despite the various methods developed to specifically measure LDL-C. This limitation is likely reflected by the differences in interference due to remnant cholesterol and the degree of interference observed among different systems. The positive interference of LDL-C with remnant cholesterol was observed in six systems, among which a similar methodology was employed in five systems. System B, C, D, E, and G all use a process in which non-LDL fractions are solubilized and removed via reactions with cholesterol esterase and cholesterol oxidase, after which only LDL-C is measured. It is presumed that residual remnant cholesterol, which is not completely removed in the non-LDL elimination step, may be the main cause of positive interference. Notably, system A and F showed no interference of remnant cholesterol, and both employ a method that selectively solubilizes only LDL using a surfactant and measures LDL-C, while surfactant and sugar compounds inhibit non-LDL to be measured. Although a positive bias caused by remnant cholesterol was observed, system H also employed a similar strategy, in which only LDL-C was measured while the block polymer prevented the measurement of non-LDL. This may explain the relatively lower correlation between the remnant cholesterol/LDL-C ratio and the LDL-C bias of system H, compared with other systems (B, C, D, E, and G). These results suggest that the method of solubilizing and measuring only LDL-C is more efficient for measuring LDL-C specifically than the two-step method, in which non-LDL-C is removed and LDL-C is later measured.

Reports show that HDL-C measurement using the direct method can be biased [21, 28–30]. Factors such as dyslipidemia, high TG levels, low HDL-C levels, and paraproteins can contribute to measurement bias [21, 29, 30]. In this study, we identified remnant cholesterol as a potential source of bias in HDL-C measurement using the direct method. Notably, the impact of remnant cholesterol varied based on the reagent used and was associated with either a positive or negative bias. As various techniques are employed for different reagents to selectively measure HDL-C [31], these methodological differences may contribute to variations in the effects of remnant cholesterol. Previous studies have revealed a positive bias in HDL-C measurement with a reagent depending on TG levels [29]. Similarly, in this study, a positive bias due to high remnant cholesterol was observed in some measurement systems. Positive interference by remnant cholesterol was observed in three systems (B, C, and D), in which a detergent was used in each to selectively solubilize non-HDL for removal before measuring HDL-C. Similar to LDL-C, incomplete removal of remnant cholesterol in the non-HDL elimination step may cause positive interference. In contrast, negative interference was observed in systems A, H, and F. The specific materials used for each differ; however, they share the common principle of measuring HDL-C after inhibiting non-HDL reactions via polyanions or block polymers. Meanwhile, the mechanism by which high remnant cholesterol levels cause a negative bias in these systems remains unclear and requires further investigation.

The bias in LDL-C and HDL-C levels caused by remnant cholesterol, as identified in this study, may affect patient classification based on LDL-C measurements. This is particularly relevant because direct LDL-C measurement and calculated LDL-C, derived from equations incorporating HDL-C values, are widely used in clinical settings. Our findings indicate that high remnant cholesterol affects both directly measured and calculated LDL-C levels. Moreover, the degree of bias varies based on the reagent. Therefore, laboratories should consider the specific reagents they use and assess the potential measurement bias associated with LDL-C values especially when remnant cholesterol is high.

Most laboratories routinely report calculated LDL-C values. While various studies have investigated the accuracy and limitations of different calculation methods [32, 33], our findings indicate that the analytical system itself may be a major source of bias in calculated LDL-C values. Since calculated LDL-C depends on the measurements of TC, TG, and HDL-C, any system-specific bias in these parameters may directly influence the result. This was evident in our data, where the calculated LDL-C values varied substantially across systems, even when using the same set of formulas on identical samples. Therefore, clinicians and laboratories should be aware that calculated LDL-C values may differ depending not only on the formula used, but also on the measurement system employed.

In this study, we used a reference method to calculate remnant cholesterol and elucidate its relationship with bias observed in direct LDL-C and HDL-C measurements. However, accurate quantification of remnant cholesterol in routine clinical laboratories remains challenging. Therefore, the TG/LDL-C and TG/HDL-C ratios can be used as alternatives to estimate the potential bias in direct measurements in routine practice.

Our study has some limitations. First, although we prepared 41 different reference materials, the relatively small number of samples made it difficult to perform proper statistical analyses for the subgroup data, which should be interpreted with caution. Second, although we included samples with a range of lipid concentrations, the variability present in the general population – including those with extremely high TG levels, severe dyslipidemia, and extremely high or low LDL-C levels – may not have been fully captured in these samples. In particular, as direct methods are often applied to samples with extreme TG levels, further studies investigating the effect of extreme TG levels and remnant cholesterol on directly measured LDL-C and HDL-C are warranted. Third, remnant cholesterol was calculated indirectly and not measured directly; thus, the accuracy of its determination may be slightly influenced. However, the TC, HDL-C, and LDL-C levels used to calculate remnant cholesterol levels were measured using the reference method. Therefore, the calculated value of the remnant cholesterol can be considered accurate. Fourth, we were unable to clearly explain the mechanism underlying the negative bias of HDL-C observed in some measurement systems. This remains an open question and should be addressed in future studies.

In conclusion, this study reveals that remnant cholesterol can serve as a significant source of positive bias in direct LDL-C measurement and both positive and negative bias in direct HDL-C measurement, which vary according to the measurement system. Clinical laboratories should be aware of this potential interference, particularly in patients with elevated remnant cholesterol levels. In addition, manufacturers of measurement systems that exhibit a notable bias associated with remnant cholesterol should prioritize refining their methodologies to minimize remnant cholesterol interference. The focus of future research should be on enhancing the current direct LDL-C and HDL-C assays or developing new methods that can more effectively discriminate LDL and HDL from remnant lipoproteins, thereby improving the accuracy and clinical utility of direct measurements.

Research ethics: In this study, we used 41 commutable frozen serum samples that were produced following the Clinical and Laboratory Standards Institute C37-A guidelines from Severance Hospital, Konkuk University Medical Center, and Chungnam National University Hospital (Institutional Review Board approval numbers: 4-2017-0351 [Severance Hospital], 2022-03-037 [Konkuk University Medical Center], and 2022-09-078 [Chungnam National University Hospital]).
Informed consent: Informed consents were obtained from all donors.
Author contributions: S.S.K. – Writing (original draft), Visualization, Resources, Methodology, Investigation, Formal analysis, Data curation; H.J. – Writing (review & editing), Resources; J.H.R – Writing (review & editing), Resources, Methodology, Investigation, Data curation; J.C. – Writing (review & editing), Resources; J.D.S. – Writing (review & editing), Resources, Methodology, Investigation, Data curation; Y.Y. – Writing (review & editing), Resources, Methodology, Supervision; Y.M.P. – Writing (review & editing), Resources; G.C.K. – Writing (review & editing), Resources; C.C. – Writing (review & editing), Resources; S.L. – Writing (review & editing), Resources, Methodology, Data curation, Conceptualization; J.L. – Writing (review & editing), Resources, Supervision. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Conflict of interest: The authors state no conflict of interest.

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Data availability: Data is available from the corresponding author upon reasonable request.

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