Removal of Inhibitory Effects in a Serum Cardiac Troponin I Immunoassay

To the Editor:

The measurement of cardiac troponin I (cTnI) has become the gold standard for the clinical diagnosis of myocardial infarction (1). Varieties of commercial sandwich-type immunoassays are used for the measurement of cTnI concentration in human serum or plasma. Several of these assays use pairs of monoclonal antihuman cTnI antibodies with high specificities for the invariant part of the cTnI molecule (amino acid residues 30–110), in accordance with the recommended guidelines (2). These assays are often based on magnetic beads or latex particles to take advantage of the ease of bead/particle washing, which can minimize assay interference by matrix proteins. Signal amplification by means of chemiluminescence or electrochemiluminescence allows the detection of serum cTnI at concentrations <0.05 µg/L.

As a part of the recent effort of the IFCC Working Group for Standardization of Troponin I to develop a cTnI reference measurement system (1), we have used various measurement techniques to investigate binding affinities between 6 monoclonal antibodies (mAbs) obtained from HyTest (each serving as either the capture or the detection antibody) and cTnI, in the form of either a reference material (NIST SRM 2921) or a cTnI-positive serum pool (PS). One of the techniques is the multiplexed bead array (3), which resembles the commercial bead- or particle-based immunoassays. The capture mAb molecules are covalently immobilized on different bead populations to capture cTnI. The bound cTnI is recognized by the biotinylated detection antibody, which is then bound to a fluorescent reporter, streptavidin–phycoerythrin (SA-PE). Although commercial cTnI assays show higher detection sensitivities, the ease of the assay multiplexing and the low cost of the instrumentation make the bead-array platform advantageous for optimization of antibody pairs for cTnI detection.

It is well known that a human serum matrix can introduce inhibitory effects that cause immunoassays to report lower concentrations of cTnI. These inhibitory effects could be due to the presence of heterophilic antibodies and/or autoantibodies in the serum (4). Finding the optimal conditions to minimize the inhibitory effects and enhance cTnI detection is crucial to obtain the most robust and accurate immunoassay results. Therefore, bead arrays were used in the testing of 18 different conditions, including depletion of serum samples before analysis with commercial antibody-based multiprotein depletion columns, magnetic beads coupled with protein G or protein A, and carboxylated beads co-valently coupled to antimouse IgG. In addition, various antihuman antibodies to serum protein (IgG, IgA, IgM, complement component C3), mouse IgG, detergent (Triton X-100), buffer solutions with different pH values (4.0–7.4), EDTA, and a combination of both buffer solution and EDTA were added separately to serum samples before the cTnI-capture step. For both SRM 2921 spiked into normal (nonpathologic) human serum (NS) and a PS, the addition of both EDTA and a buffer solution at pH 5.2 to the sample substantially diminished the inhibitory effect of the matrix on bead assays performed with different antibody pairs. An optimized bead-array assay protocol is as follows: Initial addition of 12.5 µL of a solution of 15 mmol/L EDTA in sodium acetate buffer (0.1 mol/L), pH 5.2, is followed by addition of 10 µL NS, 7.5 µL SRM 2921 spiked into PBS blocking buffer [BB in the letter refers to 0.01 mol/L PBS (0.138 mol/L NaCl, 0.0027 mol/L KCl), pH 7.4; 1% BSA; 0.05% NaN₃], and 10 µL BB. After beads (14 µL) are added and incubated for 30 min, 10 µL biotinylated mAb (8.1 × 10⁴ µg/L) is added. After 30 min, 10 µL SA-PE (1.0 × 10⁵ µg/L) is added.

The left panel of Fig. 1 shows that for SRM 2921 spiked into NS, the addition of 12.5 µL of acetate-EDTA solution to a total sample volume of 40 µL before the addition of the capture mAb–coated beads produces a significant increase in assay response (▼), compared with the same dilution of the serum in BB (▲). This increase in signal was also observed for PS (right panel). The addition of this buffer component to the serum samples increases the sensitivity of cTnI detection and enables more reliable measurement of cTnI in serum. Considering that the strong interaction between cTnI and troponin C (TnC) is Ca²⁺ dependent (5) and that the high positive charge of cTnI (pI 9.87) increases interactions with negatively charged molecules such as TnC (pI 4.05), our result suggests that cTnI in both SRM 2921 and PS is largely associated with TnC, because both a low buffer pH and EDTA likely help to reduce the strong interaction between cTnI and TnC and thereby allow binding of the capture mAb to cTnI. Although this approach has not been evaluated on com-

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1 Nonstandard abbreviations: cTnI, cardiac troponin I; mAb, monoclonal antibody; PS, cTnI-positive serum pool; SA-PE, streptavidin-phycoerythrin; NS, normal (nonpathologic) human serum; BB, PBS blocking buffer; TnC, troponin C.
commercial cTnI immunoassay platforms, we speculate that it may also be beneficial to commercial platforms for enhancing the sensitivity and reliability of cTnI detection.

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References

Lili Wang*
David M. Bunk
Hua-Jun He
Kenneth D. Cole

Chemical Science and Technology Laboratory
National Institute of Standards and Technology (NIST)
Gaithersburg, MD

* Address correspondence to this author at: National Institute of Standards and Technology 100 Bureau Dr., Stop 8312 Gaithersburg, MD 20899-8312 Fax 301-330-3447 E-mail lili.wang@nist.gov

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Cardiac troponin I (cTnI) is the gold standard biomarker for diagnosing patients with acute coronary syndromes. Expert panels have defined the decision limit at >99th percentile of a reference population and assay imprecision of ≤10%. Prototype ultrasensitive cTnI assays allow accurate detection of low concentrations of cTnI in healthy individuals. However, these novel assays require analytical and biological validation before they can be put into clinical practice.

We previously reported the 99th percentile cutoff of 7 ng/L for the Erenna® cTnI immunoassay (Singulex), with the requisite precision. Using a single-molecule counting technology, the limit of detection (0.2 ng/L) surpassed many other commercial assays. The improved limit of detection is not an artifact of nonspecific binding events (1). We also showed that the increased analytical sensitivity of the assay enabled measurement of the short- and long-term biological variation of cTnI from healthy individuals (2). However, to what extent minute levels of cTnI instability may have contributed to this variation was not established.

We determined the in vitro stability of cTnI in whole blood and serum over short- and long-term intervals in both healthy subjects and in a small subset of patients who presented to the San Francisco General Hospital Emergency Department. A protocol for the use of leftover blood from routine collections and for collecting blood of healthy subjects was reviewed and approved by the University of California San Francisco Committee on Human Health. Previous reports demonstrated cTnI stability at high concentrations in patients with cardiac injury (3–5), despite the fact that these samples may contain proteolytic enzymes that are coreleased following cardiac injury. None of these studies used high-sensitivity assays to demonstrate analyte stability at the low concentrations observed in healthy individuals. Because the Erenna cTnI immunoassay has high analytical sensitivity, our study will answer whether differences in clinical study design, such as timing between blood draws, removal of serum or plasma from cells, and subsequent specimen testing procedures, will contribute variability to cTnI measurements.

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Four milliliters of blood from apparently healthy volunteers who self-reported no cardiac symptoms was drawn into EDTA-plasma and serum separator tubes (SSTs). To assess stability over a short-term period of 6 h, 4 EDTA and 4 SST samples were collected from each of 15 volunteers. The first sample (t = 0) for each specimen type was centrifuged and immediately frozen at −80 °C. The remaining samples were left at room temperature for 2, 4, and 6 h before centrifugation and −80 °C storage. Analyte stability after 1- and 6-h room temperature storage were also assessed in 10 healthy subjects and 12 emergency department patients who presented with chest pain and had low cTnI concentrations. To assess stability over a long-term period of 48 h, we collected 3 EDTA and 3 SSTs from each of 15 volunteers. EDTA plasma samples were either centrifuged and frozen immediately or left at room temperature for 24 or 48 h before spinning and −80 °C storage. SST samples were spun first, then either frozen immediately or left at room temperature for 24 or 48 h before −80 °C storage. Resulting samples were thawed and batch tested using the Erenna cTnI immunoassay.

The mean CV was 11% (range 4%–24%) for cTnI measurements over 6 h, and 14% (range 2%–34%) over 48 h (Table 1). The variation was similar when compared to other conventional assays. The percent changes over 1 h for healthy subjects and 6 h for chest pain patients were <1% and <3%, respectively. Compared to other conventional assays, analyte variability was not different in this study. A previous experiment performed using the iSTAT assay noted <4% change in cTnI concentration between samples stored refrigerated or at room temperature over 14 days (3). A study using the Immulite system found <15% differences in samples stored with different protocols (4). However, variability among plasma and serum samples determined with the Vidas system at different temperatures was statistically significant (P < 0.0001) (5). In our analysis, the change in analyte concentration per hour during the 6-h interval was minimal [mean 0.001 (SD 0.031) ng/L]. For the long-term, 48-h interval, we noted a small positive increase in the change in analyte concentration per hour [0.015 (0.033) ng/L, or 0.339 (0.793) ng/L per day]. Variation in cTnI above the analytical precision cutoff (≥20% CV) was observed in 17% of short-term and 33% of long-term samples, suggesting that measurements are most precise when freshly prepared samples are used.

However, these limits did not exceed the reference change values of −32% and −45% calculated from the biologic variation of cTnI (2), suggesting that minor analyte in-
stability will not lead to results that are clinically relevant. Analyte stability was not affected by sample type in our study, with both whole blood–EDTA plasma and serum samples providing similar measurements.

Using serum or EDTA-plasma, low concentrations of cTnI from blood of healthy subjects and emergency department patients appears to be as stable as the high concentrations released from patients with myocardial damage. Although the mechanism of protein release may be different between normal tissue turnover of healthy subjects and that from minor ischemic injury, once cardiac troponin is in the blood itself, there are no differences with respect to stability. These data will be useful for prospective and retrospective cardiovascular studies when measuring low cTnI concentrations using high-sensitivity assays.

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References


To the Editor:

Mutation scanning by high-resolution melting analysis has been described previously (3). In brief, samples were amplified on the LightCycler® system (Roche); the amplicons were then analyzed by high-resolution melting with the HR-1™ instrument (Idaho Technology). The exon 15 reverse and forward primers were 15R1 (5’-GAGCCCCGCCTCAT-3’) and 15F1 (5’-GCTATT-3’) (3). Because the 15F1 primer has a nonpathogenic polymorphism at this position (c.2608-24G>A), samples with the wild-type allele will also amplify with the 15F1 primer, including samples heterozygous for both polymorphisms (i.e., c.2608-24G>A and c.2608-24G>G) had similar Cq values compared with the wild-type sample. Samples that were homozygous for the wild-type G nucleotide at position c.2608-24G>A were not recommended for mutation scanning, because their use would detect 2 common polymorphisms, complicating the assay results.

The protocol for RET mutation scanning by high-resolution melting analysis was described previously (3). In brief, samples were amplified on the LightCycler® system (Roche); the amplicons were then analyzed by high-resolution melting with the HR-1™ instrument (Idaho Technology). The exon 15 reverse and forward primers were 15R1 (5’-CTGG GAGCCCCGCCTCAT-3’) and 15F1 (5’-GCTATT-3’), respectively. The position of intron 14 polymorphism c.2608-24G>A in 15F1 is marked as (G). Another forward primer, 15F4 (5’-CACACACC ACCCCCTCTGCTG-3’), which does not anneal over the c.2608-24G>A polymorphism, was used with reverse primer 15R1 under the same experimental conditions. All de-identified samples were sequenced for RET exon 15, which includes the c.2608-24G>A polymorphism region [forward primer 15F4 (5’-TGCTGGTCAACCC AGGCTG-3’) and reverse primer 15RSeq (5’-TGCCCCATGTTGACCTG-3’) were used for amplification and sequencing].

To test amplification efficiency, we compared quantification cycle (Cq) values for samples homozygous for intron 14 polymorphism c.2608-24G>A with samples that had the wild-type sequence at this polymorphism position. Because samples can have inherent differences, such as DNA concentration, that affect Cq values, we also amplified the samples with a different forward primer (15F4) that is unaffected by the c.2608-24G>A polymorphism. The samples homozygous for the c.2608-24G>A polymorphism amplified with primer 15F1 had a delay of approximately 1 cycle compared with the wild-type samples or when the 15F4 primer was used. Samples that were heterozygous for both polymorphisms (i.e., intron 14 c.2608-24G>A and exon 15 c.2712C>G) had similar Cqs with the two exon 15 forward primers 15F1 and 15F4.

Because the 15F1 primer has the wild-type G nucleotide at position c.2608-24G>A, the amplicons produced from any sample with that primer, including samples heterozygous or homozygous for the c.2608-24G>A polymorphism under the 15F1 primer, will also have the wild-type G nucleotide at that position (confirmed by sequencing; data not shown). Therefore, use of the 15F1 primer actually masks the presence of the c.2608-24G>A polymorphism, thereby avoiding this complication in analyzing melting-curve data. Samples homozygous wild type for

Letters to the Editor

RET Mutation Scanning
Update: Exon 15

To the Editor:

Mutation scanning by high-resolution melting analysis has a high diagnostic sensitivity and specificity (1, 2). Our 2006 Clinical Chemistry report demonstrated that mutation scanning of RET (ret protooncogene) detects mutations causative of multiple endocrine neoplasia type 2 (3). To illustrate mutation-scanning data for exon 15, we used cell lines that had the wild-type sequence or were heterozygous for a common, non-pathogenic exon 15 polymorphism (c.2712C>G, p.S904S), which occurs at an allele frequency of 11%–27% (4, 5), but samples with other exon 15 sequence variants were not available at that time. We have re-visited this mutation-scanning assay for exon 15 because a sample with a novel exon 15 sequence variant (c.2673G>A, p.S891S) (5) became available for testing. Additionally, the published exon 15 forward primer had inadvertently been designed to anneal over a nonpathogenic polymorphism within intron 14, c.2608-24G>A (rs2472737). The variant allele for this polymorphism occurs at a frequency of 6%–28%, depending on the population (4, 5). Because this polymorphism is common and closer to the 5’ end of the exon 15 forward primer, we used samples of known genotypes to test the function of this published primer for amplification efficiency, allele dropout, and mutation-scanning results for RET exon 15.

The protocol for RET mutation scanning by high-resolution melting analysis was described previously (3). In brief, samples were amplified on the LightCycler® system (Roche); the amplicons were then analyzed by high-resolution melting with the HR-1™ instrument (Idaho Technology). The exon 15 reverse and forward primers were 15R1 (5’-CTGG GAGCCCCGCCTCAT-3’) and 15F1 (5’-GCTATT-3’), respectively. The position of intron 14 polymorphism c.2608-24G>A in 15F1 is marked as (G). Another forward primer, 15F4 (5’-CACACACC ACCCCCTCTGCTG-3’), which does not anneal over the c.2608-24G>A polymorphism, was used with reverse primer 15R1 under the same experimental conditions. All de-identified samples were sequenced for RET exon 15, which includes the c.2608-24G>A polymorphism region [forward primer 15F4 (5’-TGCTGGTCAACCC AGGCTG-3’) and reverse primer 15RSeq (5’-TGCCCCATGTTGACCTG-3’) were used for amplification and sequencing]. Fig. 1 shows the primer positions and the locations of sequence variants. Of note, the 15F4 and 15FSeq primers are not recommended for mutation scanning, because their use would detect 2 common polymorphisms, complicating the assay results.

To test amplification efficiency, we compared quantification cycle (Cq) values for samples homozygous for intron 14 polymorphism c.2608-24G>A with samples that had the wild-type sequence at this polymorphism position. Because samples can have inherent differences, such as DNA concentration, that affect Cq values, we also amplified the samples with a different forward primer (15F4) that is unaffected by the c.2608-24G>A polymorphism. The samples homozygous for the c.2608-24G>A polymorphism amplified with primer 15F1 had a delay of approximately 1 cycle compared with the wild-type samples or when the 15F4 primer was used. Samples that were heterozygous for both polymorphisms (i.e., intron 14 c.2608-24G>A and exon 15 c.2712C>G) had similar Cqs with the two exon 15 forward primers 15F1 and 15F4.

Because the 15F1 primer has the wild-type G nucleotide at position c.2608-24G>A, the amplicons produced from any sample with that primer, including samples heterozygous or homozygous for the c.2608-24G>A polymorphism under the 15F1 primer, will also have the wild-type G nucleotide at that position (confirmed by sequencing; data not shown). Therefore, use of the 15F1 primer actually masks the presence of the c.2608-24G>A polymorphism, thereby avoiding this complication in analyzing melting-curve data. Samples homozygous wild type for
the exon 15 polymorphism had identical melting curves, regardless of the presence or absence of intron 14 polymorphism c.2608-24G>A (Fig. 1). Samples homozygous for exon 15 polymorphism c.2712C>G were indistinguishable from the wild-type samples in this assay. Samples heterozygous for both polymorphisms (c.2712C>G and c.2608-24G>A) had the same melting curves as samples heterozygous for only the c.2712C>G polymorphism, indicating there was no allele dropout due to intron 14 polymorphism c.2608-24G>A. In addition, the new sample that was heterozygous for both the novel c.2673G>A variant and exon 15 polymorphism c.2712C>G was distinguished from the other genotypes (Fig. 1).

Although the 15F1 primer for the RET mutation-scanning assay was inadvertently designed to anneal over intron 14 polymorphism c.2608-24G>A, there were no substantial differences in amplification efficiency for samples homozygous for the c.2608-24G>A polymorphism, no allele dropout was detected with the heterozygous samples, and the RET exon 15 mutation-scanning result was as expected for each sample. Thus, we have demonstrated that the presence of intron 14 polymorphism c.2608-24G>A in a sample does not interfere with the exon 15 mutation-scanning assay with the published 15F1 primer and that mutation scanning detects the novel exon 15 variant (c.2673G>A).

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References

Rebecca L. Margraf*, Fernanda R.O. Calderon1
Rong Mao1,2
Carl T. Wittwer1,2

1 ARUP Institute for Clinical and Experimental Pathology
Salt Lake City, UT
2 Department of Pathology
University of Utah Medical School
Salt Lake City, UT

* Address correspondence to this author at:

ARUP Institute for Clinical and Experimental Pathology
500 Chipeta Way
Salt Lake City, UT 84108
E-mail rebecca.margraf@aruplab.com

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Limitations of Automated Remnant Lipoprotein Cholesterol Assay for Diagnostic Use

To the Editor:

I wish to comment on the limitations of an automated remnant lipoprotein cholesterol (RemL-C) assay reported in Clinical Chemistry (1). Remnants are lipoprotein particles produced after newly formed triglyceride-rich lipoproteins (TRLs) of either hepatic or intestinal origin enter the plasma space and undergo lipolysis via the action of lipoprotein lipase in the capillary bed. During this process, these lipoproteins lose triglyceride and pick up cholesteryl ester and apolipoprotein E (apoE) from other lipoproteins through the action of cholesteryl ester transfer protein. The development of a clinical diagnostic method for measuring remnant lipoprotein cholesterol has been hampered by difficulties with isolation. Moreover, the characteristics of remnant lipoproteins have not been clearly defined. The most consistent definition of remnant lipoproteins has been proposed as an apoE-rich lipoprotein fraction within TRLs, which increases in the postprandial state. The original immunoseparation method for the measurement of remnant-like lipoprotein particle cholesterol (RLP-C) was developed by Nakajima and colleagues in Japan and satisfied these criteria. Normal ranges in the US for this analyte were developed using samples from the Framingham Offspring Study (2). The separation of newly formed TRLs from remnant lipoproteins is crucial because the later lipoproteins are atherogenic and are important for the assessment of coronary heart disease (CHD) risk, especially in women.

My colleagues and I previously reported that an increased RLP-C, in contrast to triglyceride, was a significant independent predictor of prospective CHD in female participants in the Framingham Offspring Study, after adjustment for other standard risk factors including LDL and HDL cholesterol concentrations (3). In addition, in this study triglyceride values correlated with RLP-C (r = 0.79, P < 0.001), but the correlation was not strong enough to affect the clinical utility of the RLP-C assay. We also recently measured remnant lipoprotein cholesterol with the RemL-C assay as described (1) in samples from 3201 male and female participants in cycle 6 of the Framingham Offspring Study. We noted a highly significant correlation between these values and triglyceride concentrations (r = 0.93, P < 0.0001). Moreover, values obtained with this assay were not independent predictors of CHD in either a case-control or prospective fashion. These data suggest that this recently developed automated assay for remnant lipoprotein cholesterol does not accurately measure the cholesterol content of remnant lipoproteins, but rather total TRLs. This issue has taken on increasing importance since prospective studies have reported that postprandial triglyceride (TG) concentrations are associated with significantly...
higher risk of CHD than fasting TG concentrations, suggesting that such differences can be due to postprandial increases in remnant lipoproteins (4). Nakajima et al. (5) have recently reported that 80% of the increase in triglyceride concentrations in the postprandial state results from increases in remnant lipoproteins using the RLP assay. Therefore, postprandial TG may reflect higher CHD risk than fasting TG due to increased remnant lipoproteins. These data also indicate that the correlation between remnant lipoproteins and TG should be different in the fasting and postprandial states, namely a higher correlation between remnant lipoproteins and TG in the postprandial state. However, the RemL-C assay did not show such differences between TG vs fasting and postprandial RemL-C in our other studies.

These two different remnant assay methods (RLP-C and RemL-C) have been approved for the measurement of serum remnant lipoprotein cholesterol concentrations in Japan, whereas only the RLP-C assay is approved for this purpose in the US. The benefits of the newer RemL-C assay are that it does not require pretreatment and can be readily used on an automated analyzer. In my view, however, this latter assay has limitations because of its very high correlation with triglyceride concentrations and its lack of CHD risk prediction in the Framingham Offspring Study, suggesting a lack of specificity for remnant lipoproteins.

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References


Ernst J. Schaefer
Lipid Metabolism Laboratory
Human Nutrition Research Center on Aging at Tufts University, and Tufts University School of Medicine
Boston, MA

Address correspondence to the author at:
Lipid Metabolism Laboratory
Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University
711 Washington Street
Boston, MA 02111
Fax 617-556-3103
E-mail ernst.schaefer@tufts.edu

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In Reply

We appreciate the concerns from Ernst J. Schaefer on our article regarding development of a homogeneous assay for remnant lipoprotein cholesterol (RemL-C) (1). I agree that RemL-C has a limitation for a diagnostic use in a certain situation. The process of this assay includes a degradation step of lipoproteins by detergents. Freeze-thawing or long-term storage, which also degrades lipoproteins, yields a nonnegligible effect on the values measured by RemL-C. This fact limits the use of RemL-C assay to fresh samples or samples frozen and thawed only once. I am afraid that samples from the Framingham Offspring Study were measured after repeated freeze-thaw or long-term storage.

Schaefer defined remnant lipoproteins in the concerned letter as the apolipoprotein E–rich lipoprotein fraction within triglyceride-rich lipoproteins. RemL-C reagents did surely react to these lipoproteins as shown in the original article (1). Schaefer mentioned that RemL-C correlates with triglyceride (TG) too closely to be employed for clinical use. Recent reports, however, clearly demonstrated that RemL-C has value in detecting a high-risk group for atherosclerosis. Nakada et al. (2) reported that RemL-C concentrations were increased in patients with coronary artery disease independent of TG concentrations, and they concluded that the RemL-C assay has a clinical significance in assessing coronary risk, particularly among patients with normal TG concentrations. In another study, Yoshino et al. revealed that RemL-C did not always correlate with TG, especially in patients with diabetes mellitus (personal communication, G. Yoshino, 2012 Clinical Chemistry 55:11 (2009)
August 24, 2009). They found that some patients showed abnormally high concentrations of RemL-C although their TG concentrations were <100 mg/dL (1.13 mmol/L). Recently, we investigated the clinical significance of RemL-C determination in an apparently healthy population and found that the mean concentration of RemL-C was above the reference range in normotriglyceridemic patients with visceral obesity and carotid atherosclerosis (unpublished data). Furthermore, Yoshida et al. (3) reported that RemL-C correlated with remnant-like lipoprotein particle cholesterol (RLP-C) in the general population, but the RemL-C assay was likely to reflect intermediate-density lipoprotein more closely than RLP-C. I believe that Schaefer himself appreciates the clinical usefulness of RemL-C because very recently he and his colleagues reported an interesting study employing RemL-C as a marker of remnant lipoprotein metabolism (4). In that article, the effectiveness of statins on serum TG and RemL-C tended to be the same between atorvastatin and rosuvastatin, and the reduction rate of RemL-C did not parallel that of TG in both statins. These data clearly depict the independence of RemL-C from TG. From these observations, we can definitely say that the RemL-C assay has an enough specificity to identify remnant lipoproteins and can provide useful clinical information.

There is no doubt that the RLP-C assay successfully clarified the clinical significance of remnant determination (5). The efforts made by K. Nakajima and his colleagues, who developed the RLP-C assay in establishing the clinical importance of remnants, should be well considered, but it might be time for RLP-C to pass the baton to RemL-C as a routine laboratory parameter.

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**References**


Masato Ishigami

Division of Health Sciences, Osaka University Graduate School of Medicine

Suita, Osaka, Japan

Address correspondence to the author at:

1-7 Yamada-oka

Suita, Osaka 565-0871, Japan

Fax +81-6-6879-2499

E-mail ishigami@sahs.med.osaka-u.ac.jp

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