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Oxidized LDL is stable in human serum under extended thawed-state conditions ranging from −20 °C to room temperature

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ABSTRACT

Introduction: Oxidized LDL (oxLDL) is formed by the spontaneous reaction between aldehyde byproducts of lipid peroxidation and lysine residues of apolipoprotein B within LDL. Clinically, oxLDL is used as a marker of coronary artery disease and predictor of metabolic syndrome risk. Despite its popularity as a clinical marker, no systematic studies of oxLDL stability, in which serum or plasma has been pre-analytically exposed to an array of different time and temperature conditions, have been carried out.

Objective: To systematically evaluate the stability of oxLDL in human serum samples exposed to thawed conditions (> −30 °C) for varying periods of time while monitoring a second protein/small molecule redox system as a positive control for non-enzymatic biomolecular activity.

Methods: OxLDL was measured in serum samples, from 24 different humans, that had been pre-exposed to three different time courses at 23 °C, 4 °C and −20 °C using ELISA kits from Mercodia that employ the 4E6 mouse monoclonal antibody. A liquid chromatography/mass spectrometry-based marker of serum exposure to thawed conditions known as ΔS-Cys-Albumin was employed as a positive control.

Results: OxLDL was stable in serum exposed to 23 °C for up to 48 h, 4 °C for 21 days, or −20 °C for 65 days. ΔS-Cys-Albumin changed dramatically during these time courses ($p < 0.001$).

Conclusions: OxLDL is remarkably stable ex vivo in human serum samples exposed to thawed conditions.

Introduction

Oxidized low-density lipoprotein (oxLDL) is a form of LDL in which tens to hundreds of the primary amine-bearing lysine residue side chains of apolipoprotein B molecules within LDL have covalently bonded with aldehyde byproducts of lipid peroxidation, such as malondialdehyde [1–3]. In vivo, oxidation of LDL is believed to occur in arterial walls—especially those that are atherosclerotic and contain increased concentrations of copper and iron [1], which are key mediators of lipid peroxidation. OxLDL is then recognized by scavenger receptors on macrophages [4,5], which take it up. This results in the conversion of macrophages to foam cells, which are key initiators of atherosclerosis [6,7]. In addition to macrophages, smooth muscle cells can become foam cells and contribute significantly to arterial lesion development. Fatty streak formation can occur before the infiltration of leukocytes into the arterial intima of atherosclerosis-prone arteries [8–10].

Besides its role in atherosclerosis, oxLDL is involved in tumor growth and metastasis. Binding of oxLDL to its receptor, LOX-1, stimulates the expression of proteins that promote inflammation, angiogenesis, adhesion [11,12] and transendothelial migration [12]. LOX-1 is elevated in substantial percentages of patients in a wide variety of cancers [13] and is widely regarded as an important tumorigenic drug target [11,13,14]. Our particular interest in studying oxLDL in cancer patients stems from our recent finding that oxidized and desialylated LDL inhibits the anti-tumor functions of lymphokine activated killer cells [15].

The first ELISA for oxLDL in blood plasma was developed in the

Abbreviations: OxLDL, oxidized low-density lipoprotein; CAD, coronary artery disease; CHD, coronary heart disease.

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1990s [2]. Since that time, the measurement of oxLDL in plasma/serum has been carried out in numerous clinical studies [16–23] and is currently offered as a test from several clinical diagnostics laboratories [24–26] where it has been utilized as a marker for the risk of CAD [16–19,21] and of the risk of developing metabolic syndrome [23] and CHD [22], although its value as an *independent* predictor of CHD has been questioned [27].

Given that oxLDL is a downstream product of lipid peroxidation [1] and that some lipids are highly susceptible to ex vivo peroxidation in thawed plasma/serum [28–30] (i.e., plasma/serum warmer than −30 °C [31,32]), there is cause for concern about the ex vivo stability of oxLDL [2,33,34]. It has been claimed (without direct evidence) that LDL oxidation does not occur in the presence of plasma/serum [1], but given the popularity of oxLDL as a biomarker, the lack of a systematic study of its ex vivo stability in plasma or serum is surprising. A couple of studies have evaluated the stability of oxLDL in blood specimens under specific conditions, such as heparin-anticoagulated whole blood kept on cold packs for 24 or 36 h [33] or plasma/serum kept for 2 h at room temperature or 24 h in a refrigerator [34]—and in most cases it was found to be stable.

To our knowledge, however, no systematic evaluation of oxLDL stability under a series of temperatures and time ranges has been conducted. The purpose of this study was to comprehensively evaluate the stability of oxLDL in human serum over time courses at 23 °C, 4 °C, and −20 °C while simultaneously measuring ΔS-Cys-Albumin—a well characterized marker of plasma/serum exposure to thawed conditions that is based on the ex vivo oxidation of albumin to S-cysteinylated albumin [35–38].

Materials and methods

Materials

Serum was collected from cancer-free donors and gastrointestinal (GI) cancer patients under informed consent and local IRB approval by the Cooperative Human Tissue Network (CHTN; Nashville, TN). Basic donor demographics and disease status are provided in Table 1. Detailed inclusion and exclusion criteria are provided in Supplementary Material. Notably, patients with poor kidney function (eGFR < 60 mL/min per 1.73 m^2) were excluded because poor kidney function can, in theory, result in abnormally high ΔS-Cys-Albumin measurements [36]. All samples were obtained in compliance with the Declaration of Helsinki principles. Specimen receipt and analysis were approved by the Arizona State University IRB. ELISA kits for measuring oxLDL in plasma or serum were purchased from Mercodia (Uppsala, Sweden). These kits employ the 4E6 mouse monoclonal antibody developed by Holvoet et al [2] in a capture or "sandwich" format. This antibody was originally raised against "copper-oxidized LDL" [2]. As shown in the original publication, this antibody binds with greatest affinity to apolipoprotein B-100 molecules that have over 200 malondialdehye (MDA)-modified lysine residues, followed by copper-oxidized LDL which bound with about the same affinity as oxLDL from the plasma of patients with severe chronic renal failure. Native LDL bound with approximately 1,000-fold less affinity than copper-oxidized LDL [2]. In a subsequent paper, Holvoet and Collen showed that the 4E6 antibody binds almost identically to copper-oxidized rabbit LDL and rabbit VLDL as it does to human copper-oxidized LDL. Copper-oxidized rabbit HDL, however, bound roughly 1,000-fold less effectively to the 4E6 antibody [39]. Water, acetonitrile (LC/MS grade) and formic acid (ACS certified grade) were purchased from Fisher Scientific.

Table 1
Patient/donor characteristics.

Patient Demographics	n (%)
Number of Patients	24
Gender	
Males	13 (54.2)
Females	11 (45.8)
Age (yrs)	
<40	3 (12.5)
40–60	11 (45.8)
>60	10 (41.7)
Race	
White	20 (83.3)
Black	4 (16.7)
Other	0 (0)
Disease status	
Cancer-Free	12 (50.0)
Cancer	12 (50.0)
Stage I	1 (8.3)
Stage III	1 (8.3)
Stage III	1 (8.3)
Stage IV	3 (25.0)
Stage undetermined	6 (50.0)

Blood collection and related pre-analytical information

Blood was collected and processed according to a strict protocol: A standard 10-mL tube of serum was collected from each patient. Pre-evacuated collection tubes were from BD, product number BD 367820. They contained a silicone-coated clot activator, but no separator gel. Blood was drawn by forearm venipuncture using an 18- or 20-gauge needle. Tubes were properly filled then immediately inverted five times (never shaken). Tubes were allowed to clot for 45 min followed by centrifugation at 1300×*g* for 10 min at room temperature. Serum was then immediately aliquoted on ice. Aliquots were put into a −80 °C freezer within 2 h of the initial draw time. In order to verify the processing timing steps, time stamps were recorded at 1) the time of initial draw, 2) the time centrifugation was completed, and 3) the time at which aliquots were placed at −80 °C. Serum samples with visually noted hemolysis > 250 mg/dL were excluded. This was done despite the fact that the Mercodia ELISA directions for use state that grossly lipemic, icteric or hemolyzed samples do not interfere with the assay. Unless otherwise noted, all serum samples were kept at −80 °C prior to analysis.

Serum was shipped to Arizona State University (Tempe, AZ) overnight on dry ice. Once received, specimens were verified to be frozen then unpacked into a −80 °C freezer equipped with continuous temperature monitoring. All specimens were kept in the −80 °C freezer for at least 7 days prior to thawing to ensure that any residual CO_2 in the headspace was exchanged for air in order to avoid CO_2-induced sample acidification [40].

Study design

Baseline values of ΔS-Cys-Albumin and oxLDL in separate, pristine, never-thawed serum aliquots from 12 cancer-free control donors and 12 GI cancer patients were measured within a time range of 12–20 months (ΔS-Cys-Albumin) or 18–30 months (oxLDL) after initial specimen collection, during which time specimens were continually kept at −80 °C. Analyses were delayed from the time of initial collection primarily due to the fact that the specimens were collected over a period of multiple months but for practical reasons needed to be run in batches. The 24 donors selected for inclusion in the stability studies were chosen from amongst a larger population of 84 people in which baseline ΔS-Cys-Albumin measurements were made in order to ensure that the initial ΔS-Cys-Albumin values of samples included in this study were equally distributed across the entire range of initial ΔS-Cys-Albumin values measured in the 84 patients [38]. In the interest of avoiding analytical block effects, serum specimens from both clinical groups were randomized before analysis using a random number generator to create the run order assignments.

Thawed-state stability studies involved incubation of separate serum aliquots for up to 96 h at 23 °C, 28 days at 4 °C, or 65 days at −20 °C. A

separate aliquot for each individual non-baseline thawed-state ΔS-Cys-Albumin and oxLDL assay time point (either 20 μL for ΔS-Cys-Albumin or 50 μL for oxLDL measurements), was created from a parent aliquot that had never previously been thawed. To create these aliquots, the parent sample was thawed and kept on ice with strict time tracking for a period of 3.4 ± 1.1 (SD) min for the 23 °C-exposed samples, 8.5 ± 2.0 min for the 4 °C-exposed samples, and 14.2 ± 3.0 min for the −20 °C-exposed samples—by which time all temperature exposure time courses were started. Aliquots for the time courses were immediately placed in a −80 °C freezer upon completion of their time/temperature exposure period. Once all time courses were completed, all time course aliquots were randomized and ΔS-Cys-Albumin or oxLDL were measured. OxLDL at all stability time points (including baseline) was measured as a randomized set of 240 serum samples (each measured in duplicate) using Mercodia ELISA kits.

Given that the serum samples were collected from patients over a period of about one year, all oxLDL measurements were taken within 18–30 months after initial specimen collection. Except for intentional thawed-state incubation periods, all samples were continually kept at −80 °C prior to analysis. After all ΔS-Cys-Albumin and oxLDL time course aliquots were analyzed, ΔS-Cys-Albumin was again measured in a separate never-thawed aliquot of each sample in order to confirm the long term stability of ΔS-Cys-Albumin at −80 °C. This happened one year after the initial ΔS-Cys-Albumin measurements in never-thawed samples were made.

Laboratory procedures

Measurement of ΔS-Cys-Albumin: Randomized serum specimens were prepared and the percentage of albumin in the S-cysteinylated form (S-Cys-Albumin) was measured as we have described previously [35,36]. Briefly, one microliter of serum was diluted 1000-fold in 0.1 % (v/v) trifluoroacetic acid (TFA) and injected onto an LC-MS instrument where albumin was concentrated and desalted on a capillary-sized OPTI-TRAP™ cartridge designed for protein analysis, and then directly eluted into the mass spectrometer for measurement of the intact protein and relative quantification of its proteoforms. Nine microliters of the same serum sample was then put in a 0.6-mL polypropylene Eppendorf snap-cap tube and incubated in a dry oven at 37 °C for 24 h. One microliter of the incubated sample was then diluted 500-fold in 0.1 % TFA and injected onto the LC-MS for analysis. ΔS-Cys-Albumin is defined as the *difference* between the percentage of albumin in the S-cysteinylated form before and after the overnight incubation at 37 °C that drives the percentage of S-Cys-Albumin to its maximum value [36]. Additional technical details pertaining to the LC-MS method, including data processing, are provided in Supplementary Material.

Measurement of oxLDL: OxLDL was measured using Mercodia ELISA kits according to the manufacturer's instructions, with one exception: We found that the recommended dilution factor of 1/6,561 was insufficient for many samples and, therefore, opted for a 1/13,225 dilution factor. This was accomplished by two serial dilution steps of 10 μL (of serum or 115-fold diluted serum sample) added into 1,140 μL of sample buffer to give a total volume of 1,150 μL. Each sample was measured in duplicate. Each batch contained five calibrator samples (each measured in duplicate and fit with a second order polynomial regression curve without averaging any values), a low-concentration QC sample and a high concentration QC sample that were both supplied with the ELISA kit, and two aliquots of a randomly selected human plasma sample that was not supplied in the ELISA kit. In total, six ELISA kits were required for this project; each one was run on a different day.

Statistical analysis

To evaluate the stability of oxLDL in serum, the concentration at each time–temperature condition was compared with its respective control aliquot that was kept continuously at −80 °C. Data consisted of 24 subject specimens, each measured longitudinally over time at temperatures of 23 °C, 4 °C, and −20 °C. First, we performed descriptive statistics and analyzed ΔS-Cys-Albumin at baseline (time = 0), comparing the effect of health status (cancer-free vs cancer), gender (female vs male), and race (black vs white) using a two-sample *t*-test. The age effect was assessed using correlation and linear regression analyses. Next, we analyzed longitudinal ΔS-Cys-Albumin data using linear mixed effects models to account for the repeated measures within subjects. Specifically, we considered the full model as follows:

$$y_{ij} = \beta_0 + \beta_1 age_i + \beta_2 status_i + \sum_{j=1}^{q} \beta_{3j} t_{ij} + \sum_{j=1}^{q} \beta_{4j} age_i t_{ij} + \sum_{j=1}^{q} \beta_{5j} status_i t_{ij} + \epsilon_{ij}$$

where y_{ij} is the concentration of ΔS-Cys-Albumin for the i^{th} subject at j^{th} time point, age_i is the age for the i^{th} subject, $status_i$ is 1 if i^{th} subject's status is cancer and 0 otherwise, $t_{ij} = 1$ if y_{ij} is observed at the j^{th} time point, and $t_{ij} = 0$ otherwise, ϵ_{ij} is an error term, and q is the number of time points observed, excluding the baseline. We employed a step-down approach that dropped the interaction term of status and time, if they were not significant, and used reduced models with only the main effects.

The same approach was used to analyze oxLDL, with natural logarithm transformed y values to improve normality—which was achieved at nearly every time point, indicating that overall the oxLDL data distributions were not severely skewed—a condition that linear mixed models can robustly withstand [41,42]. Bonferroni-adjusted *p*-values<0.05 were regarded as statistically significant. All the analyses were conducted at each temperature separately.

GraphPad Prism® software (version 9.3.1) was employed for descriptive statistics and t-tests, and MIXED procedure in SAS 9.4 (SAS Inst. Inc., Cary, NC) was used for linear mixed effects models.

Results

Basic demographics and clinical characteristics of the patients who provided specimens for this study are provided in Table 1. OxLDL was measured in randomized human serum samples that had either been kept continuously at −80 °C since collection (Time 0/baseline) or exposed to three different time points at 23 °C, 4 °C, or −20 °C (Fig. 1, **Tables S1-S3**). For all three exposure temperatures, no significant differences were observed between baseline concentrations of oxLDL and those at any of the exposure time points. OxLDL concentrations observed in baseline serum specimens were not different between donors of different gender, race, or health status (GI cancer patients or cancer-free individuals) as determined by a two-sample *t*-test ($p > 0.05$). Neither was there a correlation of baseline oxLDL with age (Pearson correlation coefficient = -0.035, $p > 0.05$). For reference, the standard curves for each of the six ELISA plates are provided in Supplementary Material (**Fig. S1**). All but one standard curve had coefficient of determination (R^2) values>0.99; all were>0.98. The overall coefficient of variation CV for the inter-batch QC plasma sample that was run in duplicate in each batch was 18 % (not including one outlier value; Grubb's test with $\alpha = 0.10$) and the CV for both the high and low control samples included with the ELISA kits was 12–13 %.

Measurement of ΔS-Cys-Albumin in the same samples as those in which oxLDL were measured confirmed that the thawed state exposures to which the serum samples were subjected resulted in the ex vivo oxidative instability of a different protein/small molecule redox system (albumin and cysteine/cystine [36]) while oxLDL remained stable (Fig. 2). (Due to the limited availability of serum aliquots and overall cost, oxLDL was only measured at a limited subset of the time points at which ΔS-Cys-Albumin was measured. All available data are shown in Figs. 1 and 2.) Mixed effects models of the ΔS-Cys-Albumin data revealed no interactions between patient health status and time or between patient age and time. After removing these interactions from the

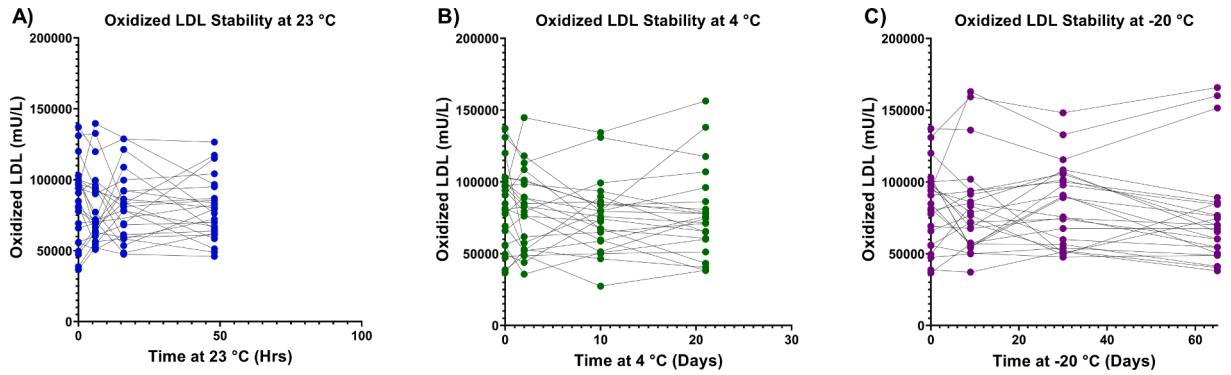

Fig. 1. Stability of oxLDL in serum over time at thawed conditions of A) 23 °C, B) 4 °C, and C) −20 °C. Serum aliquots from 12 GI cancer patients and 12 cancer-free donors were monitored at each temperature. No significant differences were observed between any thawed-state exposure time point and a single set of control aliquots kept continuously at −80 °C.

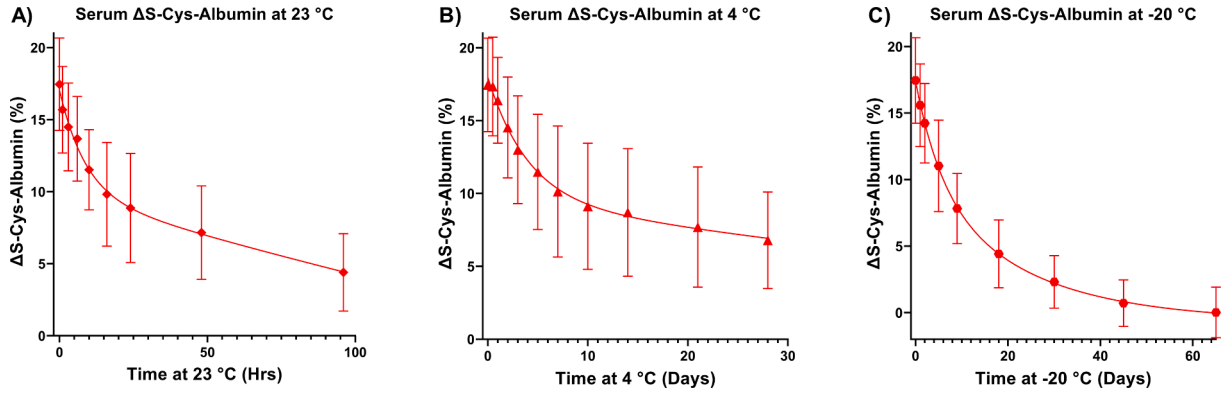

Fig. 2. ΔS-Cys-Albumin inversely reflects the oxidation of albumin to S-cysteinylated albumin [36]. Here it serves as a positive control for ex vivo changes that occur in a different protein/small molecule redox system present in serum when it is exposed to thawed conditions at A) 23 °C, B) 4 °C, and C) −20 °C. Serum aliquots from the same 12 GI cancer patients and 12 cancer-free donors shown in Fig. 1 were monitored at each temperature. Data points represent the average of all 24 individuals and error bars represent inter-donor standard deviation.

model, data were re-analyzed to focus solely on main effects. As anticipated [36], exposure time at all three thawed-state temperatures induced major changes in ΔS-Cys-Albumin (Fig. 2; Bonferroni adjusted $p < 1 \times 10^{-8}$). Simultaneous observation of these oxidative changes in the same samples in which oxLDL was measured confirmed the ex vivo stability of oxLDL under the thawed-state conditions examined. Notably, ΔS-Cys-Albumin in never-thawed serum aliquots kept continuously for an additional year at −80 °C, following the time zero measurements shown in Fig. 2, did not significantly differ from the original results (paired *t*-test, $p > 0.05$, $n = 24$; data not shown).

Discussion

Previous studies on the stability of oxLDL in serum, plasma or whole blood had pointed toward its relative stability, but were limited with regard to the scope of ex vivo conditions examined. In 2002, Pai et al showed that oxLDL in sodium heparin plasma was stable when whole blood was kept on cold packs for up to 36 h prior to processing [33]. Though speculative, this level of stability on cold packs would likely translate into at least several hours of whole blood stability at room temperature. Two years later, Perman et al [34] reported that oxLDL was stable in EDTA plasma and serum for 2 h at room temperature or up to 3 years at −80 °C. On the other hand, they reported a decrease in oxLDL when plasma or serum was kept for 24 h in a refrigerator. Notably, however, the authors did not state whether samples were randomized within or between batches, meaning that a batch effect cannot be ruled out as a possible explanation for the instability observed. Both of these reports employed the same ELISA from Mercodia as was employed in the present study.

Two limitations were relevant to this study. First, it was partially limited by the modest precision of the ELISA employed. Inter-batch CV values for the in-kit controls and an actual EDTA plasma sample were 12–13 % and 18 %, respectively. These were higher than the 8.3 % CV values reported for total assay variation by the manufacturer, although it is unclear whether this lower value reported by the manufacturer resulted from the repeated analysis of the in-kit controls or an actual plasma or serum sample. The large number of paired measurements ($n = 24$ per temperature) provided some compensation for partial loss of the ability to detect subtle changes caused by higher than expected analytical imprecision. Moreover, analyses were conducted by experienced personnel, so the precision observed here is likely to be similar to that which has occurred in the hands of others (but has not been reported). Thus, on the whole, if the precision of the Mercodia ELISA kit is good enough to facilitate use of this assay as a biomarker [22,23], then changes with disease must be substantial enough to overcome this analytical limitation—meaning that any minor instabilities masked by imprecision are essentially irrelevant at the scale at which oxLDL changes as a biomarker.

The second limitation is that this study assessed the stability of oxLDL in serum exposed to thawed conditions, but not plasma. Perman et al have reported that there are no significant differences in baseline oxLDL measurements between matched serum, EDTA plasma or heparin plasma that were freshly collected and analyzed using the Mercodia ELISA kit [34]. But inferences about the stability of oxLDL in plasma, relative to matched serum, cannot be made based on this observation.

Measurement of ΔS-Cys-Albumin in parallel aliquots of serum

provided a positive control for a second protein/small molecule redox system found in serum. Detailed analysis of ΔS-Cys-Albumin data that included matched K_2EDTA and lithium heparin plasma samples as well as higher *n*-values at time 0 has recently been published elsewhere [38]. This separate study also included an analysis of the subtle differences in ΔS-Cys-Albumin between GI cancer patients and cancer-free donors. Project costs and the available number of plasma aliquots precluded the analysis of oxLDL in matched plasma samples in this study.

Considering that this article is part of a special issue on pre-analytics, we feel it is important to point out that the U.S. Food and Drug Administration's draft guidance on biomarker qualification [43] states that "Sample collection, preparation, and storage protocols (as applicable for the biomarker type) should be established and assessed in the analytical validation studies to determine acceptability." This statement implies that as part of their validation, candidate biomarkers should undergo stability testing within their natural biological matrix. The absence of such testing—particularly when it occurs in the absence of proper pre-analytical reporting—may not only leave undiscovered pitfalls for future researchers, but can also lead to scientific irreproducibility, irreconcilable controversies and/or the reporting of false negative or (more likely) false positive results. It is fortunate that oxLDL in serum remains stable under a wide range of thawed-state conditions. In fact, since no instabilities were observed, oxLDL in serum may be stable for even longer storage periods than those evaluated here at 23 °C, 4 °C and −20 °C.

Conclusions

As measured by the Mercodia ELISA kit, oxLDL is stable in human serum that has been exposed to temperatures of 23 °C for 48 h, 4 °C for 21 days, or −20 °C for 65 days.

Ethics Statement

All samples were obtained with informed consent and in compliance with the Declaration of Helsinki principles. The Cooperative Human Tissue Network (CHTN; Nashville, TN) IRB approved specimen collection. Specimen receipt and analysis were approved by the Arizona State University IRB.

CRediT authorship contribution statement

Nilojan Jehanathan: Methodology, Investigation, Data curation. **Erandi P. Kapuruge:** Methodology, Investigation, Data curation. **Stephen P. Rogers:** Methodology, Investigation, Data curation. **Stacy Williams:** Software, Formal analysis. **Yunro Chung:** Methodology, Software, Formal analysis, Writing – original draft, Supervision. **Chad R. Borges:** Methodology, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2022.12.001.

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