recommend that the pET be used to monitor patients receiving oral enzyme replacement therapy, which is a major drawback for the clinical application of the pET.

On the other hand, the antigenic substrate in the pET seems to be equally stable in relation to elastase 1. Only elastase concentrations determined with the pET in stools stored at 4 °C for 48 h were significantly higher than directly measured concentrations. Although both pET and mET assessments were performed on stool samples stored in continuously closed cups, partial evaporation with consecutive concentration of the substrate cannot be excluded.

If we take into consideration the different aspects of fecal elastase testing highlighted in this study, the lack of specificity of the pET assay remains the crucial problem. We therefore support the proposal of renaming the polyclonal “elastase 1” ELISA because the test detects a molecule different from elastase 1 (7). Further studies should evaluate the effects of porcine enzymes on the results of the pET assay in patients with normal and mildly to moderately decreased exocrine pancreatic function. Additionally, the true antigen detected with the pET assay should be characterized to reliably define the role of this new assay in the diagnosis of exocrine pancreatic insufficiency.

Soluble CD40 Ligand Measurement Inaccuracies Attributable to Specimen Type, Processing Time, and ELISA Method, Anna Margrét Halldórsdóttir, Joshua Stoker, Rhonda Porche-Sorbet, and Charles S. Eby

CD40 ligand (CD40L) is a member of the tumor necrosis factor superfamily and is produced in a variety of cells, including platelets. The soluble form (sCD40L) is a mediator of both inflammatory and hemostasis processes and has been implicated in the pathogenesis of atherosclerosis. Clinical studies have revealed increased sCD40L in patients with unstable angina (1) and identified an association between increased sCD40L and future risk for death or nonfatal myocardial infarction (2, 3). While prospectively measuring sCD40L in a cohort of persons at risk for cardiovascular complications, we identified both preanalytical and analytical sources of error. This report documents the effects of specimen type (serum and plasma), processing (time and temperature), and commercial reagent selection on sCD40L ELISA results. These findings raise concerns about the accuracy of sCD40L results reported in recent clinical studies.

After obtaining informed consent, we enrolled 147 patients older than 60 years referred for diagnostic cardiac catheterization in an Institutional Review Board-approved study to evaluate the value of clinical, echocardiographic, and biomarker variables for prediction of future cardiovascular complications. When combined with clinical predictors, B-type natriuretic peptide and C-reactive protein, but not sCD40L, were independent predictors of death or cardiovascular hospitalization at 6 months (data not shown). The unexpectedly poor correlation between undiluted plasma sCD40L results and clinical outcomes in this study motivated us to perform the following investigations.

Venous blood from the 147 study participants [mean (SD) age, 70.8 (6.9) years] was collected into plastic tubes containing tripotassium EDTA (BD Vacutainer; Becton Dickinson) before catheterization and placed on ice for 1–4 h before processing. Blood from 10 control individuals [mean (SD) age, 38.7 (8.4) years] was collected into plastic tubes containing EDTA or nonheparinized glass tubes (Becton Dickinson) before catheterization and placed on ice for 1–4 h before processing. Blood from 10 control individuals [mean (SD) age, 38.7 (8.4) years] was collected into both EDTA and plain glass tubes (Becton Dickinson) and maintained at room temperature for 30 min before processing. Study and control samples were centrifuged twice: first at 2790g for 5 min to separate cells from the plasma/serum and then at 16 000g for 3 min to remove any residual platelets. Supernatants were aliquoted and stored at −70°C.

We assessed the effects of time and temperature on measured sCD40L concentrations by collecting whole blood from a single healthy individual into a syringe and immediately aliquoting it into EDTA-containing and plain glass tubes. For every time point analyzed, plasma and serum tubes were kept on cells at room temperature, and

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in 1 experiment they were also kept on ice. Samples were then centrifuged and stored as described above. Selected samples from the time–temperature experiment were either filtered through a 0.2 μm syringe filter or were ultracentrifuged at 200 000 g for 4 h at 4 °C to remove potential remaining platelet microparticles before repeat sCD40L testing.

sCD40L concentrations were measured with an sCD40L assay (Quantikine®; R&D Systems). According to the package insert, the R&D ELISA is suitable for measuring sCD40L in serum and plasma and is linear within the analytical range of the assay (0.0625–4 μg/L). The stated lower limit of detection is 0.0042 μg/L. Reference intervals for serum (0.675–38.373 μg/L; mean, 8.273 μg/L; n = 44) and platelet-poor EDTA plasma (0.106–11.831 μg/L; mean, 2.987 μg/L; n = 16) were provided by the manufacturer. The manufacturer’s protocols were followed.

We retested selected samples with 2 sCD40L assays (BMS 239 and BMS 293) from Bender MedSystems. The BMS 239 is suitable only for testing serum, whereas the BMS 293 is a high-sensitivity assay designed both for plasma and serum (package inserts). The lower limit of detection for the BMS 239 is 0.095 μg/L, and the lower limit of detection for the BMS 293 is 0.005 μg/L. The manufacturer’s protocols were followed.

When establishing a central 95% interval for sCD40L with 10 control plasma samples diluted 1:5, per the R&D package insert, we found that all results were below the lowest point on the calibration curve (0.0625 μg/L). Following discussions with the manufacturer’s technical consultants, we tested control and patient plasma samples undiluted. The distribution of sCD40L concentrations in undiluted plasma for the 147 patients is shown in Fig. 1A. When the 2 control and 8 patient plasma samples with sCD40L concentrations exceeding the upper limit of the calibration curve were diluted 1:2 and 1:5 in calibration diluent, the results were not linear (data not shown). To determine whether the nonlinear dilution response was a systematic analytical problem, we added recombinant sCD40L to serum and plasma samples with sCD40L concentrations <0.2 μg/L to produce a predicted concentration of 2 μg/L. Serial 2-fold dilutions of these samples in the calibration diluent also produced nonlinear results (data not shown).

Interestingly, when we compared the values for undiluted serum and plasma samples from the control group, the mean sCD40L concentration in undiluted serum (1.33 μg/L) was 6-fold higher than in undiluted plasma (0.24 μg/L). There was a weak correlation (r = 0.223) between serum and plasma concentrations for undiluted samples.

To evaluate the correlation between sCD40L concentrations measured by ELISAs from different manufacturers, we compared results obtained for selected serum and plasma samples by the R&D ELISA and the Bender BMS MedSystems ELISAs. The R&D and Bender BMS 239 assays showed good correlation for serum samples (Fig. 1B), but for plasma, the correlation was poor between the new Bender high-sensitivity BMS 293 assay and the R&D ELISA (Fig. 1C).

Finally, we investigated the impact of the processing variables time and temperature on sCD40L determinations. When serum from a healthy donor was stored on cells at room temperature, there was a 6- to 7-fold increase in sCD40L concentrations after 180 min (Fig. 1D). sCD40L concentrations in similarly treated plasma samples did not increase, and most values were below the analytical range. When either serum or plasma was stored on ice, no increase in sCD40L concentration was observed after 180 min (data not shown).

To examine whether the time-dependent increase in serum sCD40L concentrations was attributable to release of platelet microparticles producing membrane sCD40L, we either filtered or ultracentrifuged specimens before repeat testing. No difference was observed (data not shown).

Accurate measurement of an analyte is essential for its clinical diagnostic utility. Despite reports showing an association between increased sCD40L and cardiovascular complications (2, 3), there is poor agreement among studies regarding sCD40L ranges for controls or cases with similar cardiovascular risk factors (1–7). In addition, the methods sections in some reports fail to specify whether serum or plasma was tested or whether plasma was tested with an ELISA designed for testing serum, and they provide few sample-processing details (2, 3, 6, 8).

After reviewing the literature and the manufacturers’ product specifications, we decided to measure plasma sCD40L with the R&D ELISA. We were disappointed to discover that the only sCD40L ELISA suitable for plasma testing at that time lacked the sensitivity to measure sCD40L in 100% of controls when plasma was diluted 1:5, according to the manufacturer’s recommendations.

When we tested undiluted control and patient plasma samples, 10 of 157 (6%) gave exceedingly high results, and serial dilutions of these specimens produced nonlinear results. After we shared these findings with the manufacturer, the R&D sCD40L ELISA was briefly withdrawn from the market while changes were made in the assay diluent to address the presence of heterophilic antibodies in some samples. However, no changes were made to increase the sensitivity of the assay.

Most clinical studies have measured sCD40L in serum with either the R&D or Bender 293 ELISAs, reducing the problem of analytical insensitivity. Thom et al. (9) reported that mean measured sCD40L concentrations were 9-fold higher in serum than in plasma when assayed with a Bender sCD40L ELISA, which is consistent with our results. In addition, we have shown that the agreement between the R&D and Bender 239 ELISA methods for measuring sCD40L in serum was excellent (Fig. 1B).

However, we have also shown that the serum sCD40L concentration increases significantly with time in samples stored at room temperature (Fig. 1D). This is in agreement with previously published data (9, 10) and represents the combination of in vivo sCD40L, which is likely to be the
physiologically relevant component, and ex vivo-released sCD40L.
Platelets are activated during the process of clot retraction, and sCD40L shedding from the platelet surface probably accounts for the progressive increase in serum concentrations. Shed sCD40L could be bound to platelet microparticles (7). However, in our experiments, filtration and ultracentrifugation did not lead to a decrease in serum concentrations of sCD40L, suggesting that ex vivo-released sCD40L is not bound to intact membrane. It may therefore be impossible to distinguish between in vivo and ex vivo release of sCD40L.

Fig. 1. sCD40L results depend on specimen type/processing and ELISA method. (A), distribution of undiluted plasma sCD40L concentrations in 147 patients undergoing cardiac catheterization. Each data point represents 1 patient. Specimens were tested undiluted. Dotted lines represent the dynamic range of the assay. (B and C), comparison of 2 commercial sCD40L ELISA tests, using plasma and serum specimens from the time-temperature experiments. Dashed lines represent the lines of unity. Results have been corrected for dilution. (B), comparison of the R&D assay with the Bender BMS 239 for serum samples. The results of the regression analysis were as follows: $y = 0.96x + 0.42 \mu g/L$. (C), comparison of the R&D ELISA with the Bender BMS 293 for plasma samples. The results of the regression analysis were as follows: $y = 0.96x + 6.08 \mu g/L$. (D), effect of sample storage conditions on measured sCD40L concentrations. Each data point represents the mean of 2 experiments, the error bars represent SD. Serum (○) and plasma (●) samples drawn from a healthy donor were stored on cells at room temperature for different lengths of time before processing. The graph shows the change in measured sCD40L concentrations with time. Results have been corrected for dilution.
The measurement of sCD40L concentrations in human blood with the R&D ELISA is therefore problematic for the following reasons: the assay lacks sensitivity for measuring sCD40L concentrations in diluted plasma samples; testing of serum is problematic because of ex vivo release of sCD40L; there is poor correlation between plasma and serum samples; and the linearity of measurements obtained with the reformulated assay reagents has not been evaluated.

Recently, Bender MedSystems began selling a high-sensitivity sCD40L ELISA (Bender 293) suitable for plasma and serum testing. A preliminary evaluation confirmed that it is more sensitive than the R&D sCD40L ELISA test for plasma, but no further studies have been performed.

In summary, investigators should carefully consider the choice of specimen type, specimen-handling procedures, and properties of the commercial ELISA tests when measuring sCD40L concentrations in blood because each of these variables can critically affect measured sCD40L concentrations. The optimum strategy would be to measure sCD40L in platelet-free plasma by a sensitive analytical method.

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References


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Differences and Similarities between Two Frequently Used Assays for Amyloid β 42 in Cerebrospinal Fluid, Niki S.M. Schoonenboom,1,2† Ces Mulder,2† Hugo Vandersticksche,3 Yolande A.L. Pijnenburg,4 Gerard J. Van Kamp,2 Philip Scheltens,3 Pankaj D. Mehta,4 and Marinus A. Blankenstein2 (1 Alzheimer Center and Department of Neurology, and 2 Department of Clinical Chemistry, VU University Medical Center, Amsterdam, The Netherlands; 3 Innogenetics NV, Ghent, Belgium; 4 Institute for Basic Research in Developmental Disabilities, Department of Developmental Neurobiology, Division of Immunogenetics, Staten Island, NY; † these authors equally contributed to the work; * address correspondence to this author at: Departments of Neurology and Clinical Chemistry, VU University Medical Center, PO Box 7057, 1081 HV Amsterdam, The Netherlands; fax 31-(0)204440715, e-mail niki.schoonenboom@vumc.nl)

Amyloid β 42 (Aβ 42) concentrations in cerebrospinal fluid (CSF) are used to identify Alzheimer disease (AD) (1), but reported concentrations differ among studies, as does diagnostic accuracy (2). These differences may relate to the patient and control groups studied (3), processing and storage methods (4), intra- and interassay variation of the assays, or to the reagent antibodies used. A recent metaanalysis (2) stressed the importance of standardizing assays for Aβ–42 in CSF. In most studies, CSF Aβ42 was reported to be decreased, but in 2 studies, CSF Aβ 42 was not significantly changed in AD (2), and in 1 study (5) even increased in the early stages of disease. These dissimilarities might reflect the specificities of the antibodies incorporated in the assays.

The first aim of our study was to compare Aβ42 concentrations measured by 2 different assays in the same CSF samples. The first assay, widely used in Europe (6), uses 2 monoclonal antibodies (mAbs) and detects the full-length Aβ 42 peptide, Aβ 1–42 (7). The second assay [Aβ(N–42)], used mainly in the United States (8), detects both full-length Aβ 42 and Aβ peptides truncated at the NH₂ terminus (9).

The second aim of our study was to compare diagnostic accuracies of the assays for patients with AD compared with controls without dementia and patients with frontotemporal lobar degeneration (FTLD).

Finally, we investigated the relationship between CSF Aβ 1–42 and Aβ(N–42) concentrations and albumin ratio, age, disease duration, and disease severity.

Between October 2000 and December 2002, we recruited 39 AD patients, 24 FTLD patients, and 30 non-dementia controls at the Alzheimer Center of the VU University Medical Center (VUMC). All patients underwent a standardized investigative battery (3). A diagnosis of “probable” AD was made according to the NINCDS-ADRDA criteria (10); the clinical picture of FTLD (including frontotemporal dementia, semantic dementia, and progressive aphasia) was based on international clinical diagnostic criteria (11). Disease duration was defined as the time in years between the first symptoms by history and the lumbar puncture.