Fully Automated Ultrasensitive Digital Immunoassay for Cardiac Troponin I Based on Single Molecule Array Technology

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BACKGROUND: The association between increases in cardiac troponin and adverse cardiac outcomes is well established. There is a growing interest in exploring routine cardiac troponin monitoring as a potential early indicator of adverse heart health trends. Prognostic use of cardiac troponin measurements requires an assay with very high sensitivity and outstanding analytical performance. We report development and preliminary validation of an investigational assay meeting these requirements and demonstrate its applicability to cohorts of healthy individuals and patients with heart failure.

METHODS: On the basis of single molecule array technology, we developed a 45-min immunoassay for cardiac troponin I (cTnI) for use on a novel, fully automated digital analyzer. We characterized its analytical performance and measured cTnI in healthy individuals and heart failure patients in a preliminary study of assay analytical efficacy.

RESULTS: The assay exhibited a limit of detection of 0.01 ng/L, a limit of quantification of 0.08 ng/L, and a total CV of 10% at 2.0 ng/L. cTnI concentrations were well above the assay limit of detection for all samples tested, including samples from healthy individuals. cTnI was significantly higher in heart failure patients, and exhibited increasing median and interquartile concentrations with increasing New York Heart Association classification of heart failure severity.

CONCLUSIONS: The robust 2-log increase in sensitivity relative to contemporary high-sensitivity cardiac troponin immunoassays, combined with full automation, make this assay suitable for exploring cTnI concentrations in cohorts of healthy individuals and for the potential prognostic application of serial cardiac troponin measurements in both apparently healthy and diseased individuals.

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Serial measurement of cardiac troponin concentrations is an essential step in the diagnosis of acute myocardial infarction (AMI)5 (1). In addition, cardiac troponin concentrations measured shortly after AMI are highly predictive of future adverse cardiac events (2, 3). Over the last 2 decades, earlier and more reliable detection of increases in troponin following myocardial ischemia and the ensuing improved diagnostic utility of cardiac troponin assays has been largely achieved by improvements in their sensitivity and analytical performance. Although the continuously increasing sensitivity is gradually reaching a point of diminishing returns for diagnosis of AMI in the emergency department (4), there is growing interest in high-sensitivity and ultrasensitive measurement of individual baseline cardiac troponin concentrations and of more modest cardiac troponin increases in nonacute situations.

Even increases in troponin at lower concentrations are an independent long-term predictor of all-cause mortality (5). Cardiac troponin increases have been shown to have clinical significance for conditions other than acute coronary syndrome (ACS), including cardiovascular risk stratification in pulmonary embolism (6, 7), chronic heart failure (8, 9), stable type 2 diabetes mellitus (10), stable coronary artery disease (11), and chronic renal insufficiency (12), as well as combined morbidities such as anemia, type 2 diabetes mellitus, and chronic kidney disease (13, 14).

Monitoring of cardiac troponin as measured by high-sensitivity assays is gaining acceptance for the prevention of cardiotoxicity associated with certain pharma-
cotherapeutic regimens, particularly those used for cancer chemotherapy (15, 16). Similarly, detection of even small cardiac troponin increases is an early sign of rejection of a transplanted heart and, consequently, serial cardiac troponin monitoring may guide timely modifications of immune suppression in heart transplant recipients (17). Because cardiac troponin is such an effective indicator of cardiac damage, it has been used for preclinical safety drug testing in animal models (18). In these nonacute situations, analytical sensitivity and reproducibility are critical for reliable detection of small cardiac troponin increases indicative of incipient myocardial damage.

Interest has also been growing in the utility of ultrasensitive cardiac troponin measurement for primary care prevention in asymptomatic individuals. In the Dallas Heart Study, detectable cTnT among 3546 asymptomatic participants was found to be an independent predictor of heart disease and all-cause mortality (19). In the longitudinal Cardiovascular Health Study, a gradual increase in cTnT observed in serial testing was associated with higher risk for the development of heart failure and cardiac death among a community-based cohort of 4431 participants across a median follow-up of 11.8 years (20). Among the findings, a cTnT increase of >50% was associated with increased risk of heart failure and cardiovascular death, whereas a cardiac troponin decrease was associated with risk reduction in these endpoints.

A comparative assessment of the utility of 3 cTnI assays in primary prevention—based screening of asymptomatic individuals was addressed in the 14-year FINRISK (Financial Risk Institute) and BiomaCaRE (Biomarker for Cardiovascular Risk Assessment in Europe) studies, in which 7899 participants were tested using a medium-sensitivity assay (STAT Troponin I, Abbott Diagnostics), a high-sensitivity assay (ARCHITECT STAT highly sensitive Troponin I, Abbott), and an ultrasensitive cTnI assay (Erenna cTnI, Singulex) (21). Baseline cTnI was found to be a significant predictor of cardiovascular events, and risk prediction was strongest using the ultrasensitive assay, which was able to measure baseline cTnI in 93.9% of the tested population, compared with 26.4% and 81.5% for medium- and high-sensitivity assays, respectively.

Several classifications of cardiac troponin assays based on their sensitivity have been proposed (22–24). We recently suggested a simple classification because the already outdated low-sensitivity assays and medium-sensitivity assays that are still used in the US are being replaced by high-sensitivity assays, in most other countries, and ultrasensitive assays (25). A high-sensitivity assay should be able to quantify cardiac troponin in at least 50%, and ideally in 100%, of controls. Performance of the high-sensitivity assays will be adequate for early detection of acute events. Analytical performance of the ultrasensitive assays, all of which are investigational at this time, is sufficient for monitoring of other conditions, establishing individual baselines, and very early detection of even minor myocardial damage.

In addition to the assay described here, 2 additional assays that can be classified as ultrasensitive have been described. One of them, the Nanosphere assay (26), is not currently available commercially. The second method, the Singulex Erenna cTnI, relies on formation of immunosandwich complexes on a solid phase, which are chemically dissociated and passed through a laser for digital counting (27).

Recently we reported a novel, fully automated digital immunoassay analyzer based on single molecule array (Simoa) technology (28). Array technology enables single molecule sensitivity with rapid data acquisition and multiplexing using conventional ELISA reagents. Here we describe development and preliminary validation of an investigational research-use-only digital immunoassay for cTnI and its clinical performance in a cohort of heart failure patients and healthy controls.

Materials and Methods

APPARATUS
The Simoa HD-1 is a fully automated digital immunoassay analyzer that uses Simoa technology for isolation and counting of single molecules. The instrument pipettes sample directly from sample tubes (capacity 96 tubes) and processes immunoassays and data reduction with a steady state throughput of 66 tests/h. Details of the instrument have been described previously (28).

ASSAY PRINCIPLE AND PROTOCOL
The very high sensitivity enabled by Simoa technology has been discussed previously (29). In brief, conventional analog immunoassays are conducted in relatively large reaction volumes (50–100 μL) that dilute reporter molecules during the signal generation step. This dilution limits sensitivity to the picomolar range. In a Simoa digital immunoassay, dilution of reporter molecules is drastically reduced by confining individual labeled immunocomplexes to 40-fl wells. When a single molecule of enzyme label converts substrate to a fluorescent product, the fluorophores are confined to the well, creating detectable signal in a short period of time. With a camera field of view encompassing >200,000 microwells in an array, thousands of single-molecule signals in the array can be counted simultaneously. The counting of “active” and “inactive” wells constitutes a digital signal corresponding to the presence or absence of single enzyme labels. This extreme sensitivity permits use of low quantities of labeling reagent, which lowers nonspecific interactions and dramatically increases the signal-to-background ratio.
The Simoa Troponin-I assay is a fully automated 2-step sandwich immunoassay for human cTnI in serum or plasma analysis with the Simoa HD-1 analyzer. In the first step, 42 μL of sample are drawn from the sample tube by the instrument pipettor, and sample, anti-cTnI–coated paramagnetic capture beads, and biotinylated detector antibodies are combined in a reaction cuvette and incubated for 12 min. cTnI molecules present in the sample are captured by the anti-cTnI–coated capture beads and labeled with biotinylated detector antibodies. Following the incubation, capture beads are collected with a magnet, and then washed. After washing, a conjugate of streptavidin-β-galactosidase (SβG) is mixed with the capture beads for the second assay step, during which SβG binds to the biotinylated detector antibodies, resulting in enzyme labeling of captured cTnI. Following a second wash, the capture beads are resuspended in a resorufin β-D-galactopyranoside substrate solution. Digital processing occurs when beads are transferred to the Simoa array disc (28). Individual capture beads are then sealed within microwells in the array. If cTnI has been captured and labeled, the β-galactosidase hydrolyzes the resorufin β-D-galactopyranoside substrate into a fluorescent product that provides the signal for measurement. At a low cTnI concentration, the percentage of bead-containing wells in the array that have a positive signal is proportional to the amount of cTnI present in the sample. At a higher cTnI concentration, the total fluorescence signal is proportional to the amount of cTnI present in the sample (28). The concentration of cTnI in unknown samples is interpolated from a standard curve obtained by 4-parameter logistic regression fitting. Total time to first result is 45 min. Although the Simoa Troponin-I assay is currently an investigational assay for measurement of low-abundance cTnI in nonacute settings, the HD-1 instrument is designed to include acute clinical applications with random access stat capability and shortened assay protocols (28).

REAGENTS
We developed 4 reagents: paramagnetic troponin capture beads, biotinylated detector, SβG conjugate, and sample diluent. The capture beads comprised a mouse monoclonal IgG1 antitroponin antibody (SDIX) directed to amino acid residues 24–40 of the cTnI subunit. The antibody was covalently attached by standard carbodiimide coupling chemistry to 2.7-μm carboxy paramagnetic microbeads (Agilent Technologies). The antibody-coated beads were diluted to a concentration of 2 × 10⁶ beads/mL in Tris with a surfactant and BSA. Biotinylated detector reagent comprised a mouse monoclonal IgG1 antitroponin antibody (Hytest) directed to amino acid residues 86–90 of the cTnI subunit. The antibody was biotinylated using standard methods (EZ-Link NHS-PEG4-Biotin, Life Technologies) and diluted to a concentration of 2.85 μg/mL in Tris with BSA, EDTA, surfactants, and an antimicrobial. SβG was prepared by covalent conjugation of purified streptavidin (Thermo Scientific) and βG (Sigma) using standard coupling chemistry and diluted to 150 pmol/L in a phosphate buffer with a surfactant and BSA. Sample diluent was formulated in Tris with heterophilic blockers and surfactants.

CALIBRATION
We calibrated the assay using NIST human cardiac troponin complex SRM 2921. Assay calibrators were prepared by dilution of the NIST cTnI solution in Tris with BSA, mouse IgG, and surfactants. Calibrators were prepared in a logarithmic series from 0.1 to 300 ng/L. Recovery studies (below) indicated that use of this calibrator base gave a reasonable approximation to human serum. The calibration range to 300 ng/L is suitable for reading low-abundance cTnI and the vast majority of chronic heart failure patients but is not ideal for acute patients with very high cTnI, which can be well into the microgram per liter range.

CLINICAL SAMPLES
Under institutional review board approval, clinically stable patients with advanced heart failure but without ACS or hemodynamic instability who were referred to a tertiary cardiac center for device implantation or pretransplant workup underwent in-hospital imaging and clinical and laboratory assessment and were prospectively followed for occurrence of the combined endpoint of death, urgent heart transplant, or implantation of a left ventricular assist device. As part of the initial evaluation, fasting serum and EDTA-anticoagulated plasma were collected during the diagnostic hospital stay or before device implantation, and aliquots were stored at −80 °C until the time of testing. No significant assay bias was observed between serum and EDTA plasma samples in previous testing (not shown). A control set of 97 serum samples from healthy individuals (47% male, 53% female) was obtained from Bioreclamation IVT. Ages of individuals in the control group ranged from 19 to 49 years, with mean, median, and interquartile ranges (IQRs) of 31.6, 29, and 25–38 years, respectively. Racial composition was 25% white, 32% Hispanic, and 43% African American. The control group was presumed free of heart disease. Sample aliquots were stored and handled the same as heart failure samples. Although the assay is not intended for use in acute settings in which cTnI can be very high (e.g., AMI), we confirmed that the assay does not exhibit false-negative results due to high-dose hook effects by separately assaying commercially sourced AMI samples (Bioreclamation) ranging to 50 μg/L. All results were appropriately “out-of-range” (not shown).
SAMPLE HANDLING AND MEASUREMENT OF TROPONIN

Samples were thawed, vortexmixed, and centrifuged at 9000g for 10 min before assay. Samples were assayed in duplicate. Calibration was performed by running calibrators in triplicate and storing the curve in the HD-1 to be used for multiple batch runs. To avoid using a single calibration for the entire study, generally 2–3 batches of 96 samples were run with a single stored calibration curve, followed by recalibration.

STATISTICAL METHODS

Precision data were analyzed with a fully nested ANOVA design in Minitab (Minitab, Inc.). Statistical analyses of sample data used GraphPad Prism 5 statistical software.

Results

DOSE RESPONSE, LINEARITY, AND RECOVERY

Fig. 1 shows a representative dose response across a 4.5-log range. The low background typical for Simoa immunoassays is highlighted in Fig. 1A. In a study of 20 calibration curves, the mean signal-to-background ratio at 0.1 ng/L cTnI was 4.54 (SD, 0.58), indicating the presence of an additional assay range at even lower concentrations. Linearity, conducted per CLSI protocol EP6-A (30), was evaluated with admixtures of serum supplemented with NIST cTnI complex and a normal serum (Fig. 1B). Recovery of spiked cTnI from serum was within 11% of expected in samples following supplementation with high concentrations of potential interfering substances [20 mg/dL (342 mmol/L) bilirubin, 1000 mg/dL (11.3 mmol/L) triglycerides, 12 g/dL (120 g/L) protein, 500 mg/dL (77.6 mmol/L) hemoglobin].

SENSITIVITY

The limit of detection (LoD) was estimated as 2.5 SDs above background. Historically 2, 2.5, and 3 SDs above background have been employed for this purpose, whereas contemporary investigational methods employ 2.5 SDs. The LoD was calculated for each of 26 calibration runs on 2 instruments from triplicate measurements of the zero calibrator and the lowest cTnI-containing calibrator (0.1 ng/L). The mean LoD was 0.010 ng/L (SD, 0.0053 ng/L). The limit of quantification (LoQ) was estimated from dose CVs obtained from assaying diluted serum samples in replicates of 3 across multiple days and instruments (16 runs, 144 determinations). The resulting CV profile is depicted in Fig. 2. The estimated LoQ was the concentration of cTnI corresponding to a 20% dose CV. From the equation of the power fit, the LoQ was calculated as 0.079 ng/L (SE, 0.034–0.039 ng/L).

PRECISION

We assessed reproducibility per CLSI Protocol EP5-A (29), except that 5 days of testing (2 runs/day) were conducted rather than 20 days of testing. Two serum samples and 1 plasma sample were assayed in replicates of 3 twice a day for 5 days using a single stored calibration curve and a single lot of reagents. The data and nested ANOVA are depicted in Fig. 3. Within-run, between-run, and between-day CVs were <10% for all levels. The
plasma sample exhibited higher imprecision than the 2 serum samples. Rather than being due to a matrix phenomenon or the lower concentration of cTnI (2.1 ng/L) in this sample, the higher imprecision was a result of 1 replicate lying somewhat below the population of the remaining 29 replicates (Fig. 3, Run 4), which increased the within-run variance component for this data set. The total CV for the sample reading 5.8 ng/L was 6.0%. This sample was below the 99th percentile of 8.4 ng/mL, as estimated in the next section.

CLINICAL SAMPLES
EDTA-anticoagulated plasma from a total of 362 patients was tested. The average New York Heart Association (NYHA) class was 2.7 (0.6), 84% of patients were males, and in 54% of patients the heart failure was of ischemic etiology. cTnI was detected in 100% of heart failure patients, with a median concentration of 15.3 ng/L (IQR, 7.5–34.2; range 0.44–1770), as well as in all controls with median cTnI of 0.65 ng/L (IQR 0.38–1.12; range 0.07–8.40). The distribution of cTnI values in controls is shown in Fig. 4. The 99th percentile (obtained using GraphPad Prism statistical analysis) was 8.4 ng/L. A comparison of cTnI values from controls and heart failure patients is shown in Fig. 5. Both sets of data were non gaussian by Shapiro-Wilk and D’Agostino and Pearson normality tests ($P < 0.0001$) and exhibited a statistically significant difference ($P < 0.0001$, 2-tailed Mann–Whitney) between the 2 cohorts. There was an increase in cTnI concentrations with increasing NYHA class (Fig. 6); however, the trend did not reach statistical significance. No statistically significant difference in cTnI concentrations was found between patients with ischemic and nonischemic etiology of heart failure.

Discussion
We report the development of a fully automated, digital cTnI immunoassay that uses Simoa technology. This assay, with anLoD of 0.01 ng/L, LoQ of 0.08 ng/L, and CV of 10% at 2.0 ng/L, fulfills the criteria for an ultrasensitive cTnI assay in that it can detect cTnI well below the typical concentrations seen in healthy individuals. Indeed, the lowest cTnI concentration detected in the study samples, 0.072 ng/L, markedly exceeded the LoD of the assay. Among the control samples only 2 (2.1%) were below the LoQ.

The analytical performance of the assay compares favorably with that of the Abbott high-sensitivity cTnI assay that is clinically available outside the US and has a limit of blank (LoB) of 0.7–1.3 ng/L and LoD of 1.1–1.9 ng/L (31). In this multicenter method evaluation, the LoQ of the Abbott assay ranged from 4.6 to 8.1 ng/L and the cTnI concentration measured with 10% CV was 5.6 ng/L. In another study of the Abbott high-sensitivity cTnI assay, the authors observed a 10% CV at 7.3 ng/L (32). The LoD for our assay, 0.01 ng/L, is more than an order of magnitude lower than the LoD(0.2 ng/L) of the...
other ultrasensitive assay, the Singulex Erenna cTnI assay. Based on back-interpolation of calibration curves, the 10% CV of the Erenna assays was reported between 0.8 and 1.6 ng/L. (27) Although direct comparisons between concentrations of cTnI and cTnT are difficult, the LoB and LoD of the Roche high-sensitivity cTnT assay, another high-sensitivity assay clinically available outside the US, are also considerably higher at 3 ng/L and 5 ng/L, respectively. (33) Compared to the other investigational assays, the assay presented here is fully automated, with a time to first result of 45 min. Other investigational ultrasensitive assays are also more laborintensive and typically yield the first results in a few hours.

We verified the clinical applicability of the assay in 2 ways. First, we demonstrated that the assay can quantify cTnI concentrations in 97.9% of healthy individuals. Second, we measured cTnI concentrations in a cohort of heart failure patients and confirmed that these concentrations differed significantly \( P < 0.0001 \) from concentrations in the healthy group. We further correlated cTnI concentrations with disease severity as classified by the functional NYHA classification and found that the median troponin concentrations increase with increasing NYHA heart failure class. We should point out that the cTnI concentrations in our cohort of heart failure patients were markedly increased compared to those in the healthy individuals. Consequently, cTnI concentrations in the heart failure patients could have been measured in the majority of individuals by use of the high-sensitivity cardiac troponin assays that are currently available clinically outside the US. This again confirms the diminishing return of the very high-sensitivity assays like ours in patients with ACS and many other conditions associated with markedly increased cardiac troponin concentrations. In particular, this investigational assay is not intended for diagnosis of AMI, in which cTnI can be very high and easily measured by conventional immunoassays. At the same time, the exquisite sensitivity and precision of these ultrasensitive assays makes them suitable for measuring minor increases, such as those seen after
exercise stress testing or for exploring ranges of concentrations that are currently considered appropriate for a given age and sex.

Numerous reference intervals and 99th percentile cutoffs for various cTnI assays have been reported, often more than one for the same assay owing to the use of different reference populations. These reference intervals are typically quite wide and the 99th percentiles are relatively high. This may lead to a situation in which a patient’s cTnI concentration increases severalfold against his or her baseline but the increased cTnI concentration is still within the reference range for that particular assay. Such increases may remain unnoticed for extended periods of time during which the ongoing cardiac damage may become irreversible.

Ability to establish an individual’s baseline and monitor it over time may prove important, because multiple publications have demonstrated that minor increases in cTnI, even within the reference interval, can be predictive of adverse cardiovascular outcomes. Using the high-sensitivity Abbott cTnI assay, Bohula May and coworkers observed a gradient of risk for cardiovascular death or MI at 30 days after non-ST-elevation ACS in individuals having cTnI concentrations within the sex-neutral reference interval, i.e., <26 ng/L (4). Similarly, using the Singulex cTnI assay, Bonaca et al. reported increasing risk of cardiovascular death or MI 1 year after non-ST-elevation ACS with increasing concentrations of cTnI even below the method’s 99th percentile of 9 ng/L (3).
In conclusion, the very high sensitivity and full automation of the reported assay make it suitable not only for measurement of cTnI concentrations in apparently healthy individuals and for establishing personalized reference intervals, but also for detection of changes in cTnI concentrations after exercise stress testing, for monitoring of patients receiving cardiotoxic medications, for monitoring of heart transplant recipients for early signs of graft rejection, and in other applications requiring high precision and sensitivity as well as short assay time with walk-away capability.

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