



Analytical Characterization of an Enzyme-Linked Immunosorbent Assay for the Measurement of Transforming Growth Factor β 1 in Human Plasma

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Background: The transforming growth factor β (TGF- β)-signaling pathway has emerged as a promising therapeutic target for many disease states including hepatocellular carcinoma (HCC). Because of the pleiotropic effects of this pathway, patient selection and monitoring may be important. TGF- β 1 is the most prevalent isoform, and an assay to measure plasma levels of TGF- β 1 would provide a rational biomarker to assist with patient selection. Therefore, the objective of this study was to analytically validate a colorimetric ELISA for the quantification of TGF- β 1 in human plasma.

Methods: A colorimetric sandwich ELISA for TGF- β 1 was analytically validated per Clinical and Laboratory Standards Institute protocols by assessment of precision, linearity, interfering substances, and stability. A reference range for plasma TGF- β 1 was established for apparently healthy individuals and potential applicability was demonstrated in HCC patients.

Results: Precision was assessed for samples ranging from 633 to 10822 pg/mL, with total variance ranging from 28.4% to 7.2%. The assay was linear across the entire measuring range, and no interference of common blood components or similar molecules was observed. For apparently healthy individuals, the average TGF- β 1 level was 1985 ± 1488 pg/mL compared to 4243 ± 2003 pg/mL for HCC patients. Additionally, the TGF- β 1 level in plasma samples was demonstrated to be stable across all conditions tested, including multiple freeze-thaw cycles.

Conclusions: The ELISA described in this report is suitable for the quantification of TGF- β 1 in human plasma and for investigational use in an approved clinical study.

IMPACT STATEMENT

Inhibition of the TGF- β -signaling pathway is being developed for several disease states including hepatocellular carcinoma. The complexity of the pathway requires careful patient selection, and TGF- β 1 is a rational choice as a biomarker because it is the most prevalent circulating isoform of the superfamily. A colorimetric TGF- β 1 ELISA was analytically validated via assessment of assay performance, analyte stability, and applicability in hepatocellular carcinoma patients. The TGF- β 1 ELISA met the performance and quality specifications for laboratory medicine, which suggests the test will be a valuable tool for investigational use in an approved clinical study.

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The transforming growth factor- β (TGF- β) superfamily of cytokines is evolutionarily conserved and, in mammals, includes 3 TGF- β ligands: TGF- β 1, TGF- β 2, and TGF- β 3 (1). These 3 ligands are all synthesized as a polypeptide that is proteolytically cleaved into a large N-terminal latency-associated peptide and a shorter C-terminal peptide that is the active subunit (2). Additionally, TGF- β s 1, 2, and 3 are homologous with the active subunits, sharing 71%–79% amino acid identity (1). Although all 3 TGF- β ligands appear to have redundant functions *in vitro*, TGF- β 1 is the most prevalent isoform, and deficiencies of TGF- β 1 result in severe effects (3, 4). In addition to the lethal inflammation observed in knockout mice, TGF- β 1 has a wide variety of biological effects, including inhibition of cell proliferation, regulation of T-cell differentiation, immune-suppression, and regulation of the extracellular matrix (5–8). The different biological effects elicited by a single ligand are a function of receptor expression on diverse cell types and various signaling processes. Because TGF- β 1 primarily functions to maintain homeostasis, dysregulation of signaling pathways can lead to disease progression.

Therapeutic agents that inhibit TGF- β signaling are being tested for clinical efficacy in several disease states. In cancer, inhibition of TGF- β -signaling targets the tumor microenvironment to prevent progression via multiple mechanisms including inhibiting metastasis and reducing immunosuppression. Hepatocellular carcinoma (HCC), renal cell carcinoma, melanoma, glioblastoma, pancreatic carcinoma, and breast cancer are tumor types that are currently being evaluated for treatment with TGF- β -signaling inhibitors (9–14). Fibrosis of tissues such as lungs, heart, and kidneys is also associated with increased TGF- β -signaling, likely due to excess ligand availability (15). Aberrant

TGF- β -signaling may also play a role in autoimmune diseases such as scleroderma, as well as diabetes and obesity (16, 17).

Although inhibition of the TGF- β -signaling pathway is a promising therapeutic approach for many disease states, the diverse roles of this pathway in maintaining homeostasis require careful selection and monitoring of patients receiving TGF- β -signaling inhibitors. Ideally, patient selection and monitoring would be achieved through the identification of noninvasive biomarkers. Previous attempts to identify patients with TGF- β pathway activation measured the downstream signaling molecule p-SMAD2 in peripheral blood mononuclear cells (PBMCs) (18). Despite the utility of measuring PBMC responses in the context of TGF- β inhibition, the medical laboratory applicability of the method is limited due to the requirement for *ex vivo* PBMC stimulation. Therefore, an assay measuring a plasma biomarker that fits into the typical clinical laboratory work flow may be more broadly applicable for the selection and/or monitoring of patients receiving TGF- β -signaling inhibitors.

In pancreatic cancer and HCC, patients with high TGF- β 1 plasma levels had worse prognosis. In both tumor types, treatment with galunisertib, a TGF- β RI inhibitor, resulted in a reduction of plasma TGF- β 1 and a better outcome for patients (19–22). Here, we describe the analytical profile of an ELISA for measuring TGF- β 1 levels in human plasma and provide an example of clinical applicability in HCC.

MATERIALS AND METHODS

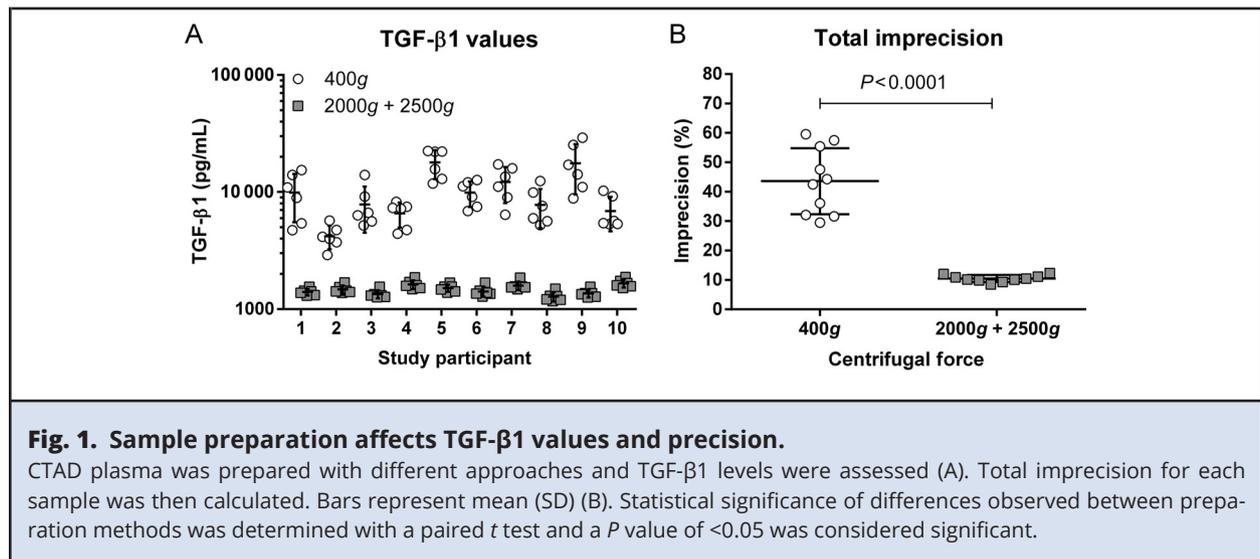
Blood draw

Venous blood was collected from study participants by standard venipuncture techniques. An additive-free Vacutainer was used to initiate blood

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[§] **Nonstandard abbreviations:** TGF, transforming growth factor; HCC, hepatocellular carcinoma; PBMC, peripheral blood mononuclear cell; CTAD, citrate-theophylline-adenine-dipyridamole; OD, optical density; CLSI, Clinical and Laboratory Standards Institute; LOQ, limit of quantitation; ECM, extracellular matrix; LTBP, latent TGF- β -binding proteins.



flow and discarded; the tourniquet was removed, and blood was collected into CTAD (citrate-theophylline-adenine-dipyridamole) Vacutainer™ tubes (Becton, Dickinson and Company). Samples were gently inverted 3–4 times to mix and placed in an ice bath for 15–30 min. Samples were then centrifuged under various conditions to separate plasma, and plasma was transferred to fresh cryovial tubes. Samples were frozen at $\leq -20^{\circ}\text{C}$ for a minimum of 24 h before analysis per the Instructions for Use.

ELISA

TGF- β 1 levels were measured by a colorimetric ELISA test kit (Corgenix Medical Corporation). The kit is only available as an IUO label and must be used in an approved clinical protocol. Briefly, 50- μL samples were treated for 2 h with 25- μL 1N HCl to activate native TGF- β 1, followed by neutralization with 25- μL 1.2N NaOH. Activated samples were then diluted, added to the precoated microwell plate in duplicate, and incubated for 1 h. Following washing, horseradish peroxidase-conjugated detection antibody was added to the plate and incubated for 1 h. Unbound detection antibody was washed off, substrate was added to the plate, and

color change was stopped after 30 min by the addition of stop solution. The absorbance (OD) of the colorimetric reaction was measured by a microplate reader (Molecular Devices) at 450 nm with a correction at 540 nm. The OD₄₅₀ signal was directly proportional to the amount of TGF- β 1 in the sample, and concentrations of TGF- β 1 were determined by interpolation against an unweighted 4-parameter standard curve prepared from serial dilutions of the recombinant-active TGF- β 1 calibrator provided with the kit (see Fig. 1 in the Data Supplement that accompanies the online version of this article at <http://www.jalm.org/content/vol3/issue2>).

Precision

Precision of the TGF- β 1 ELISA test kit was evaluated per Clinical and Laboratory Standards Institute (CLSI) Guideline EP5-A3 (23) following a $20 \times 2 \times 2$ model on each of 3 lots of kits. Thirteen independent sample pools were prepared to span the range of the assay, dispensed into single use aliquots, and frozen at -70°C . Within-run and total analytical precision were calculated with the CLSI dual-run precision evaluation test (23).

Linearity

Linearity evaluation of the TGF- β 1 ELISA was performed on kit Lot A per CLSI Guideline EP6-A (24). CTAD plasma pools representing high and low TGF- β 1 levels were used to prepare admixtures before sample activation. Samples were assayed in triplicate, and linearity was assessed by linear regression of the observed vs expected TGF- β 1 values.

Interferences and cross-reactivities

Interference of common blood components, drugs, and chemically similar molecules was assessed per CLSI Guideline EP7-A2 (25). High levels of each substance were added to CTAD plasma sample pools that spanned the range of the assay, and control CTAD plasma sample pools were matched to each substance by addition of equal volumes of appropriate solvent/buffer. Substances tested include triglycerides in saline (3000 mg/dL; Lee Biosolutions), hemoglobin in water (1000 mg/dL; Sigma Aldrich), unconjugated bilirubin in DMSO (30 g/L; Sigma Aldrich), aspirin in ethanol (4.34mM; Sigma Aldrich), acetaminophen in water (1324 μ mol/L; Sigma Aldrich), morphine sulfate in water (1.75 μ mol/L; Cerilliant), furosemide in 0.1M sodium hydroxide (181 μ mol/L; Sigma Aldrich), spironolactone in DMSO (1.44 μ mol/L; Sigma Aldrich), omeprazole in DMSO (17.4 μ mol/L; Sigma Aldrich), latent TGF- β 1 complex in PBS (50 ng/mL; R&D Systems), TGF- β 1.2 in PBS (50 ng/mL; R&D Systems), TGF- β 2 in PBS (50 ng/mL; Gibco), TGF- β 3 in PBS (50 ng/mL; Peprotech), TGF- β RI in PBS (50 ng/mL; R&D Systems), TGF- β RII in PBS (50 ng/mL; R&D Systems), TGF- β RIII in PBS (50 ng/mL; R&D Systems), TGF- α in PBS (50 ng/mL; Peprotech), and rheumatoid factor in serum (500 U/mL; US Biologicals). Interference was assessed by assay of 16 replicates of both test and control samples and calculation of the difference between the recovered TGF- β 1 values.

Reference interval

A normal reference interval was established by use of the TGF- β 1 ELISA test kit per CLSI Guideline C28-A3C (26). Reference values were obtained from 119 apparently healthy donors equally distributed from 3 collection sites: ZenBio, Plasma Services Group, and Bioreclamation.

Sample stability

Stability of TGF- β 1 in CTAD plasma was assessed for both storage conditions and freeze-thaw cycles. For storage condition stability, samples representing the range of the assay were stored at ambient temperature, 2–8 °C, –15 °C to –20 °C, and –70 °C. Samples from each storage condition were assayed at various intervals with the –70 °C stored samples representing the control condition. For freeze-thaw cycle stability, frozen samples representing the range of the assay were allowed to thaw at ambient temperature and then refrozen at –70 °C for at least 12 h for 1 cycle. The process was repeated up to 4 additional times to prepare samples exposed to 1–5 freeze-thaw cycles. After all cycles were complete, TGF- β 1 levels were assessed with a single assay.

Clinical relevance

Venous blood was collected from patients with refractory solid tumors who signed informed consent and entered 1 of 2 phase II trials studying galunisertib in hepatocellular carcinoma (*NCT01246986* and *NCT02178358*). An additive-free Vacutainer was used to initiate blood flow and discarded; the tourniquet was removed; 2-mL blood was collected into discard tube, and then 5-mL blood collected into second syringe and added gently to a CTAD tube. Samples were gently inverted 3 times to mix and placed in an ice bath for 15–60 min. Samples were centrifuged at 2500g to separate plasma, then plasma was transferred to a fresh tube and immediately frozen at $\leq -20^\circ$ for shipment to a central laboratory for testing.

Clinical trials were approved by the independent Institutional Review Boards at study sites in accordance with the Declaration of Helsinki, and all patients properly consented consistent with Good Clinical Practices.

Statistics

Data were analyzed with GraphPad Prism (GraphPad Software) and R (27). Linearity and age stratification were assessed by linear regression analysis. Differences between groups were assessed by Student *t*-test, and a $P \leq 0.05$ was considered significant.

RESULTS

Sample preparation effects

Platelet activation is a major contributor to serum TGF- β 1 levels, and contaminating platelets in a plasma sample could increase observed levels of TGF- β 1. Sample preparation effects were tested by attainment of sequential tubes of CTAD plasma from volunteers ($n = 10$) and separation of matched plasma by different methods: a single centrifugation at 400g or an initial centrifugation at 2000g, followed by a second centrifugation at 2500g. Each plasma sample was then tested over 6 independent assays to evaluate sample preparation effects on TGF- β 1 values and precision (Fig. 1). The single centrifugation at 400g resulted in increased TGF- β 1 levels compared to samples prepared with the initial centrifugation at 2000g followed by a second centrifugation at 2500g (Fig. 1A). Sample preparation also affected the precision of TGF- β 1 recovery, with the 2-step approach having an improved precision profile compared to the single-step approach (Fig. 1B). Because of the improved precision, the two-step approach was used to prepare additional samples.

Analytical performance

Precision of the TGF- β 1 ELISA test kit was assessed by testing 13 CTAD samples spanning the range of the assay in duplicate twice a day over 20 days with 3 independent lots of kit materials ($n = 240$ measurements per sample). Pooled within-run CVs ranged from 3.7% to 8.2%, while the pooled total CV ranged from 7.2% to 28.4% (see Table 1 in the online Data Supplement). Acceptance criteria were established before study execution per consultation with the Food and Drug Administration. Although all samples demonstrated within-run variability that was within the acceptance criteria, total variance for level 2 (736 pg/mL) was outside of the acceptance criteria (Fig. 2A). Linearity of the TGF- β 1 ELISA test kit was assessed by preparation of admixtures of high and low CTAD plasma samples and tested on a single lot of kit materials. The assay was found to be linear between 568 and 14255 pg/mL, with a slope of 1.010 ± 0.018 , *y* intercept at -42.26 ± 155.9 , and residuals standard deviation of 451.5 (Fig. 2B). The limit of quantification (LOQ) was estimated with a functional sensitivity method outlined in CLSI that used the precision profile at the low end of the assay range. For this estimation, the accuracy target is based solely on the precision target (20% total CV) and yielded an estimated LOQ of 1063 pg/mL.

Interference and selectivity

Interference of common blood components, drugs, and similar molecules was assessed by adding substances into CTAD plasma samples spanning the range of the assay. TGF- β 1 levels were determined and compared to control samples, with interference defined as the difference in recovered values exceeding assay repeatability for a given level. None of the common blood components or drugs tested resulted in assay interference in samples above the LOQ (Fig. 3 and data not shown). Additionally, the assay was selective for TGF- β 1 with only TGF- β 1.2, a heterodimer of TGF- β 1 and TGF- β 2 not found in

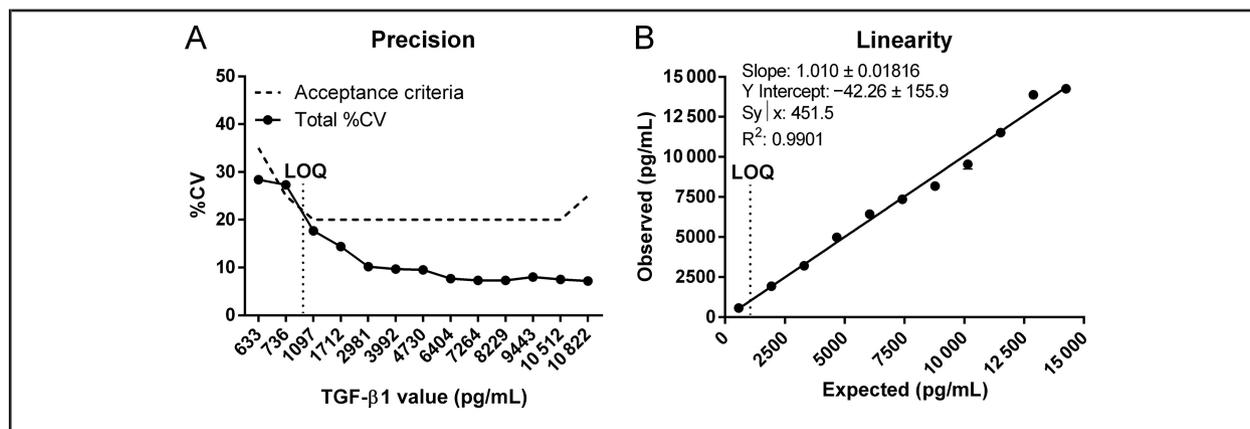


Fig. 2. Analytical performance of the Corgenix TGF-β1 ELISA.

Total %CV of each sample was compared to the expected assay performance of each level (acceptance criteria) (A). Observed recovery of TGF-β1 was plotted with the expected values and subjected to linear regression analysis (solid line) (B). Vertical dotted lines represent the estimated limit of quantitation (LOQ) at 1063 pg/mL.

humans (28, 29), resulting in differences between test and control samples greater than the assay repeatability (Fig. 3 and see Fig. 2 in the online Data Supplement). Assay repeatability was prospectively defined for each sample level as part of the testing protocol and is a function of assay precision and preparation uncertainty (23).

Reference interval

To determine the normal range of TGF-β1 levels in the population, 119 individual CTAD plasma samples were obtained and tested on 3 lots of kit materials. The reference interval of the samples tested was 1111–4542 pg/mL (Fig. 4A). The 119 samples included 60 males and 59 females with an ethnic distribution that included 40 African Americans, 41 Caucasians, and 38 Hispanics, and an age distribution of 18–63 years old (mean: 37). For the total population, the mean (SD) TGF-β1 level was 1985 ± 1488 pg/mL (Fig. 4A). Sex stratification analysis (Fig. 4B) did not indicate a significant difference between males (1735 ± 364 pg/mL) and females (2240 ± 2058 pg/mL). Equivalent levels of TGF-β1 were recovered for African Americans (1746 ± 475 pg/mL), Caucasians (1727 ± 394 pg/mL), and

Hispanics (2456 ± 2412 pg/mL) (Fig. 4C). Linear regression analysis of donor ages resulted in a slope of 2.297 ± 11.11 that did not significantly deviate from zero ($P = 0.8366$; Fig. 4D). Taken together, none of the variables tested (sex, ethnicity, and age) resulted in significant effects on TGF-β1 levels.

Analyte stability

Stability of TGF-β1 in CTAD plasma was assessed for both storage condition stability and freeze-thaw tolerance (Fig. 5). For storage condition stability testing, samples spanning the range of the assay were stored at various temperatures and assayed at temperature-specific intervals. In all the storage conditions tested, TGF-β1 levels were within 15% of the day 0 test, indicating robust stability of the analyte in matrix (Fig. 5, A–C). Additionally, freeze-thaw cycles did not affect TGF-β1 levels through the tested cycles, with each sample recovering within 15% of the day 0 value (Fig. 5D).

Clinical relevance

34 HCC patients from 2 trials (24 from JBAS, and 10 from JBAK) were used to determine the range of TGF-β1 levels in the HCC population. The 34

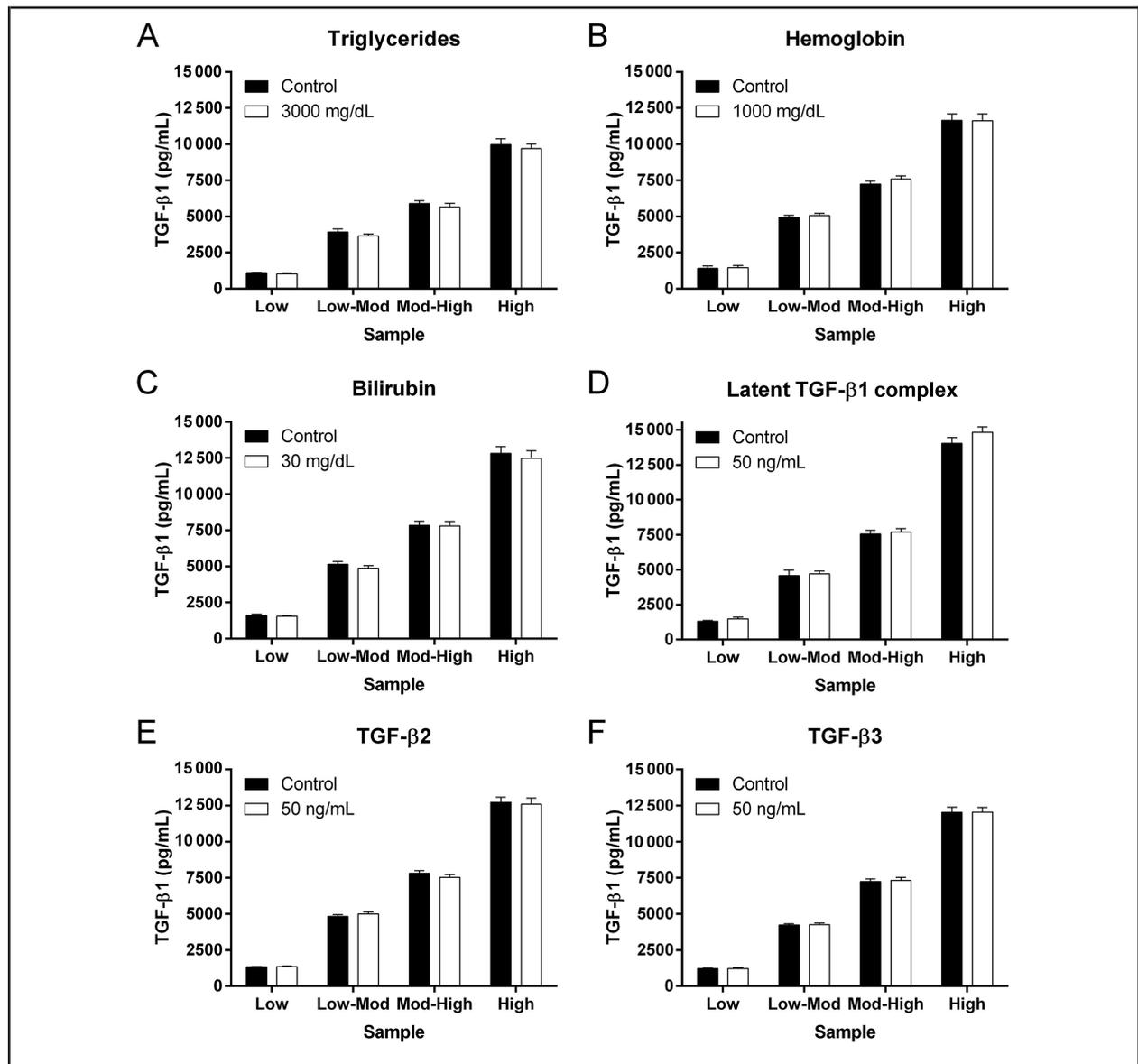


Fig. 3. Interference and selectivity of the Corgenix TGF-β1 ELISA.

Interference of common blood components (A, B, C) and similar molecules (D, E, F) was assessed by addition of 50 ng/mL of interferent into CTAD plasma samples. Controls were prepared by adding an equal volume of solvent or buffer matched to the specific interferent being tested. Bars represent the mean + standard deviation.

samples included 29 males and 5 females, with an ethnic distribution that included 24 Asian, 3 Caucasians, and 7 Missing (not reported), and an age distribution of 35–83 years (mean, 60). For the total population, the mean (SD) TGF-β1 level was 4243 ± 2008 pg/mL (Fig. 6A). Sex stratification

analysis (Fig. 6B) did not indicate a significant difference between males (4275 ± 2090 pg/mL) and females (4057 ± 1617 pg/mL). Equivalent levels of TGF-β1 were recovered for Asian (4143 ± 2277 pg/mL), Caucasians (4480 ± 1227 pg/mL), and Missing (4485 ± 1287 pg/mL) (Fig. 6C). Similarly, etiology of

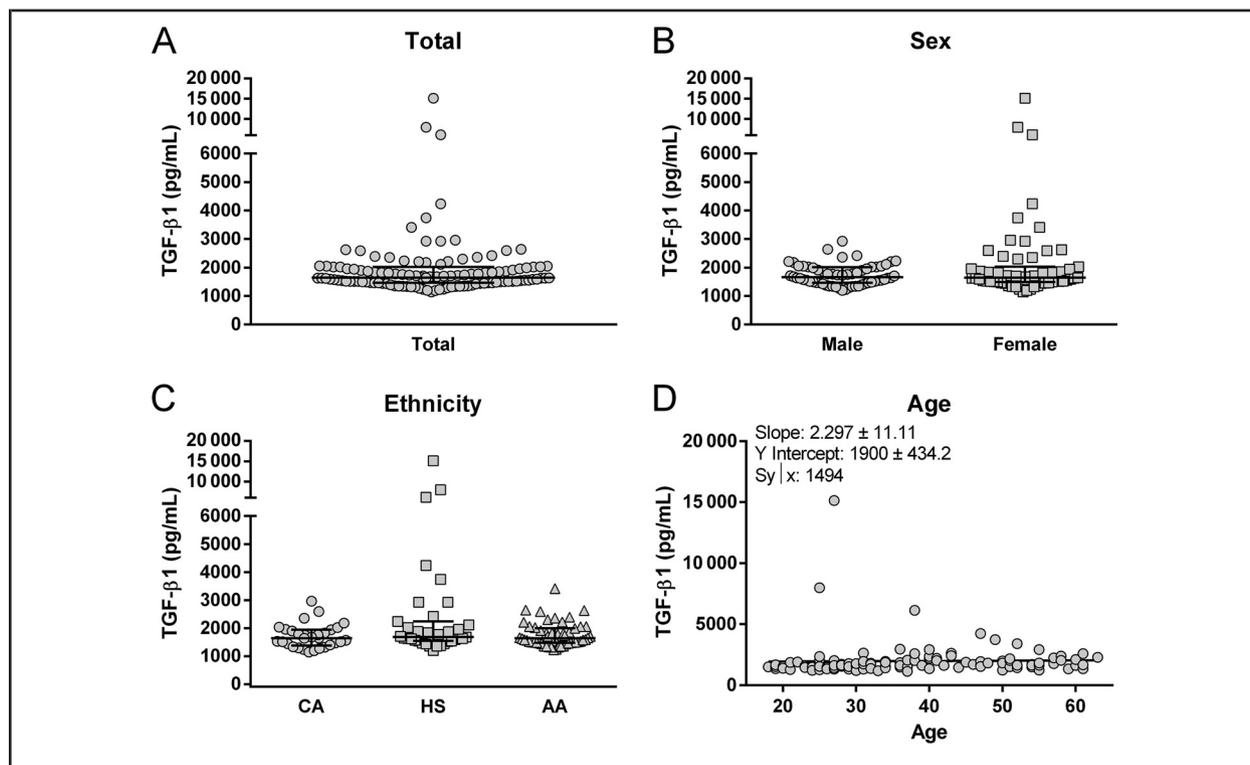


Fig. 4. Reference interval of the Corgenix TGF-β1 ELISA.

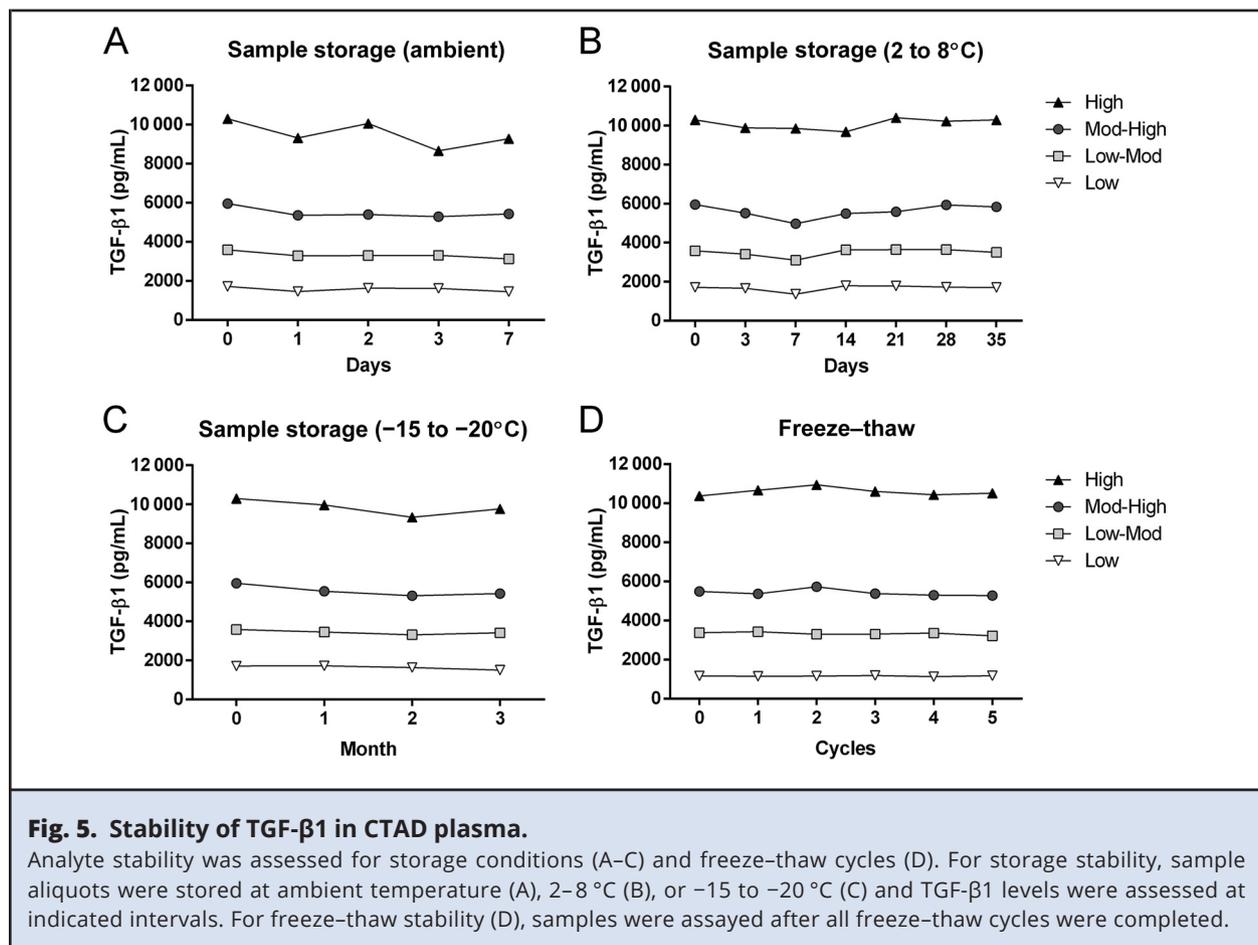
TGF-β1 levels from 119 study participants were analyzed as a total population (A) and stratified by sex (B), ethnicity (C), and age (D). Ethnicity stratification included individuals of Caucasian (CA), Hispanic (HS), and African-American (AA) descent. Bars represent median \pm interquartile range (A, B, C). Age stratification was analyzed by linear regression.

HCC did not have a significant effect on TGF-β1 levels, with all risk factors analyzed (hepatitis B, hepatitis C, and alcoholic liver disease) having equivalent levels (Fig. 6D). Taken together, none of the variables tested (sex, ethnicity, and etiology) resulted in significant effects on TGF-β1 levels within HCC patients.

DISCUSSION

The TGF-β signaling pathway is implicated in several disease states, and numerous therapeutic approaches are in various stages of development (30). Because of the various biological effects of TGF-β signaling, an assay to reliably measure circulating TGF-β1 would be an important tool to assist with patient prognostic assessment, patient selection, or patient monitoring during treatment with a

TGF-β inhibitor. The data reported here describe the analytical validation of a sandwich ELISA for the determination of TGF-β1 levels in human CTAD plasma and demonstrate that the TGF-β1 ELISA meets the performance and quality specifications for laboratory medicine. CTAD tubes were used in this study because, compared to traditional plasma collections, CTAD reduces platelet activation and is suggested to provide improved measurement of an individual's TGF-β1 levels (31). Assay precision was determined for samples ranging from 633 to 10822 pg/mL (Fig. 2), with total variance ranging from 28.4% to 7.2%. The TGF-β1 ELISA was found to be linear across the entire range of the assay (568–14255 pg/mL; Fig. 2). Importantly, no interference of common blood components or cross-reactivity with similar human



molecules was observed in samples above the LOQ (Fig. 3). Reference range testing identified the interval to be 1111–4542 pg/mL and suggested that there is no effect of sex, ethnicity, or age on TGF- β 1 levels of apparently healthy individuals (Fig. 4). Interestingly, 3 outlying individuals were identified, and the reason for the increased levels requires additional study and/or patient information that is not available. Additionally, the TGF- β 1 levels of HCC patients were increased when compared to the apparently healthy individuals that defined the reference range of the assay (Fig. 6). Taken together, the TGF- β 1 ELISA described in this report is a robust assay that could be investigated for assisting with selecting patients for treatment with therapeutic agents targeting the TGF- β –signaling pathway.

Accuracy studies were not performed during the course of this work. Although laboratory-developed tests for human TGF- β 1 are available, none of the available methods uses CTAD plasma as the sample. CTAD and EDTA plasma collected from the same donor at the same time point results in increased EDTA plasma TGF- β 1 levels (31 and data not shown). Additionally, the presence of the latency-associated peptide complicates spike recovery as the measured analyte is a fraction of the added analyte. For the assay described in this report, the calibrator value was experimentally assigned by the National Institute for Biological Standards and Control (NIBSC) TGF- β 1 standard (NIBSC code 89/514). NIBSC standards are used to ensure test results are commutable between testing locations. Given the challenges listed above,

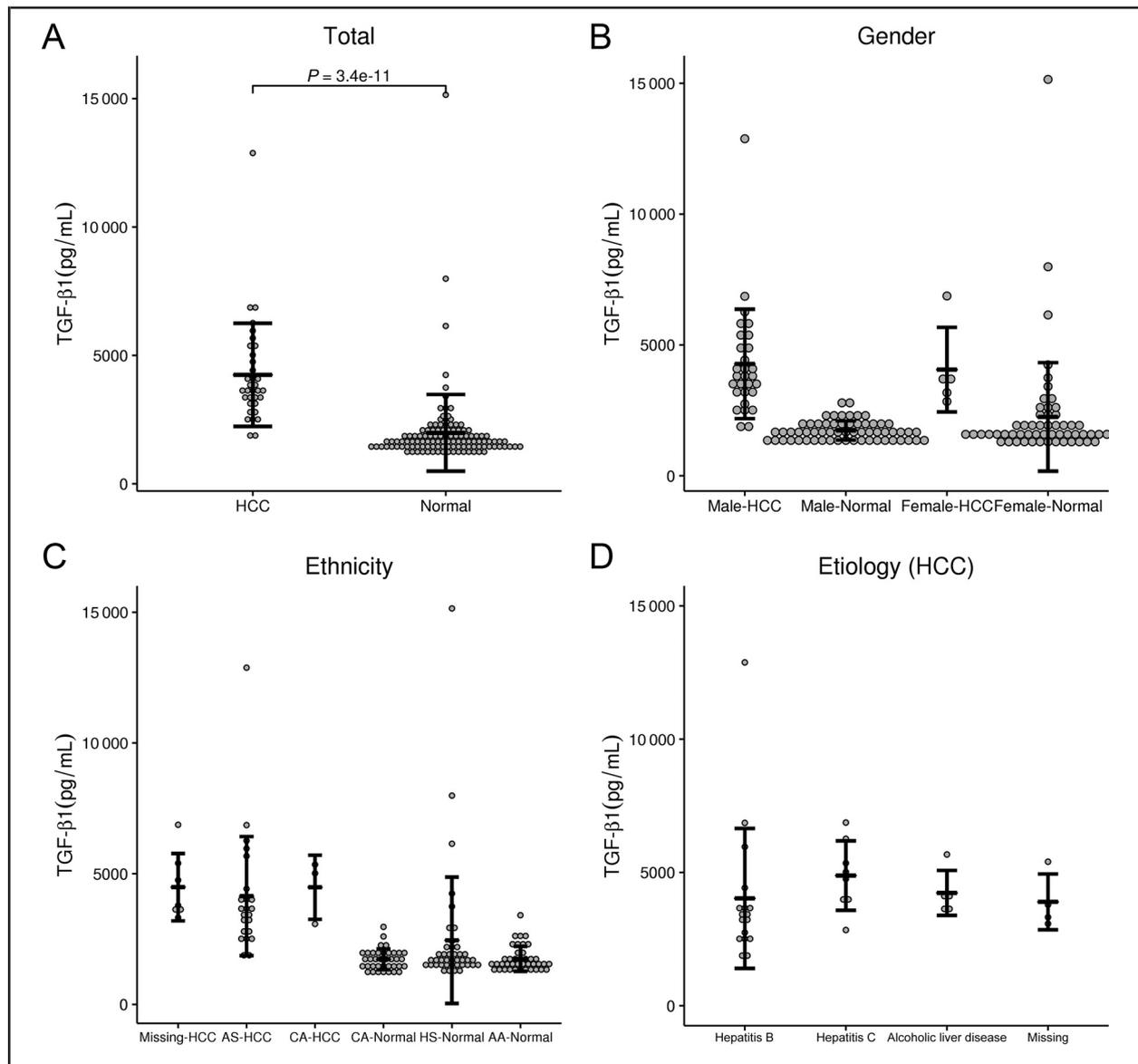


Fig. 6. Clinical relevance of TGF-β1 levels.

CTAD plasma from HCC patients was assessed for TGF-β1 levels: (A) mean ± SD of HCC and normal individuals (P value from t test), (B) mean ± SD stratified by sex, (C) mean ± SD stratified by ethnicity (AS, Asian; CA, Caucasian; HS, Hispanic; AA, African American), (D) mean ± SD stratified by etiology for HCC patients.

NIBSC TGF-β1 could be used as an analyte to establish the accuracy of similar assays in the future.

Stability of TGF-β1 in plasma was assessed for both storage conditions and freeze-thaw cycles (Fig. 5). Surprisingly, plasma TGF-β1 levels remained stable for all conditions tested. One possible

explanation for the observed stability is that active TGF-β1 is a 24-kDa homodimer that is joined by 3 interchain disulfide bonds, which could provide a structural basis for the observed stability profile (7). A second possibility is that structural features of the latent TGF-β1 complex present in human

plasma may improve stability of the active component. Plasma TGF- β 1 is noncovalently bound to the latency-associated peptide, which the solved crystal structure has revealed to completely encircle the active component (32). While this structure is expected to enforce biological latency of TGF- β 1, another effect could be to improve stability of the active molecule. Importantly, the stability profile observed in the experiments above describes the stability of the epitopes recognized by the assay antibodies and did not include assessment of biological activity.

Another unexpected finding was the effect of sample preparation on both TGF- β 1 levels and precision, with a single, slower centrifugation step being associated with increased TGF- β 1 levels and increased imprecision (Fig. 1). The most likely explanation for these observations is that the 2-spin approach removes platelets from plasma more effectively. Platelets contain reservoirs of TGF- β 1 that are released upon activation, and contaminating platelets would be expected to increase TGF- β 1 levels (33). Although TGF- β 1 reservoirs in contaminating platelets may explain the increased levels, the connection to increased imprecision is less clear. One possibility is that platelets were not equally distributed during the sample aliquoting process, leading to aliquots of the same sample with inconsistent degrees of platelet contamination. Another explanation is that along with platelets, the single-spin approach failed to remove other components of the extracellular matrix (ECM). In addition to binding TGF- β 1, the latency-associated peptide also binds to latent TGF- β -binding proteins (LTBPs), and

these LTBPs can then form covalent complexes with matrix proteins (34). Contaminating ECM components could include TGF- β 1 bound within LTBP complexes, resulting in increased TGF- β 1 levels. Additionally, variability of the LTBP-ECM complexes could also affect activation kinetics, which could contribute to increased imprecision. Proper sample preparation, therefore, is a critical factor in the performance of the TGF- β 1 ELISA.

The TGF- β superfamily of cytokines is involved in a wide variety of biological processes and has emerged as an attractive therapeutic target. Indeed, inhibition of TGF- β signaling is being developed as a therapeutic approach for several disease states including, but not limited to, cancer, fibrosis, scleroderma, and Marfan syndrome (30). The importance of TGF- β signaling makes the pathway both an intriguing and biologically complex target that requires careful and rational patient selection. Potential approaches for selecting patients include evaluation of circulating biomarkers, peripheral blood mononuclear cell responses (18), and genetic analysis (35). Because TGF- β 1 is the most prevalent circulating member of the superfamily (1), it is a rational choice as a biomarker to assist with patient selection. In conclusion, the TGF- β 1 ELISA described in this report is a candidate for further evaluation in an approved clinical trial and could become a valuable tool to help select and/or monitor patients for the emerging class of therapeutics that inhibit TGF- β signaling.

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