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# Poor agreement between commercial ELISAs for plasma fetuin-A: An effect of protein glycosylation?

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ABSTRACT

*Background:* Fetuin-A is a circulating inhibitor of ectopic calcification. Low plasma levels have been associated in some studies with increased vascular calcification, aortic stiffness and mortality in patients with Chronic Kidney Disease (CKD). However, there are other studies examining the association of fetuin-A with vascular parameters and mortality, which do not show these associations. These conflicting data may be explained by methodological differences.

*Methods:* We compared plasma fetuin-A measurements made with two widely-used commercial fetuin-A ELISA kits (Biovendor, Modrice, Czech Republic; Epitope Diagnostics Inc., San Diego, US) in samples from patients with and without CKD. We evaluated the effect of differences in fetuin-A glycosylation status on assay specificity.

*Results:* Deming regression analysis showed poor agreement between methods (for CKD cohort: $y = -0.05 + 2.52x$, $S_{y|x} = 0.099$ g/L, $R^2 = 0.694$). The Epitope Diagnostics kit demonstrated significant positive bias and greater specificity for deglycosylated fetuin-A relative to the Biovendor assay.

*Conclusion:* The apparently contradictory nature of reports of the association of fetuin-A with biological variables may reflect differences in the specificity of different ELISA methods for glycosylated plasma fetuin-A.

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## 1. Introduction

Human fetuin-A (also known as $\alpha_2$-Heremans–Schmidt glycoprotein) is a 60 kDa glycoprotein which is secreted by the liver into blood plasma [1]. Fetuin-A is a member of the cystatin superfamily of cysteine protease inhibitors and like other Type 3 cystatins has extensive post-translational modifications [2]. Fetuin-A has very complex biology and is purported to function in a diverse array of physiological process. Recent work has focussed on its role as a systemic inhibitor of ectopic mineral deposition, particularly in the setting of chronic kidney disease (CKD) where patients suffer from a heavy burden of early and progressive arterial calcification. Widespread soft-tissue calcification is seen in fetuin-A knockout mice with renal impairment or fed a mineral rich feed [3] and in fetuin-A/apoplipoprotein E-deficient mice, fetuin-A has also been found to protect against atherosclerotic calcification [4].

We recently reported that lower plasma fetuin-A concentrations were a strong and independent predictor of progressive aortic stiffness in cohort with stage 3 and 4 chronic kidney disease (CKD) [5]. However, reports in similar cohorts are not congruent with our results [6,7]. We sought to explain these discordant data, with the hypothesis that gycosylation of the native protein may be important to the biological action of the protein and that available ELISA assays may differ in their specificity for different glycosylated forms. In the setting of stage 3 and 4 CKD, reported concentrations differ by over 0.5 g/L [5,6,8]. In-house methods, based on indirect ELISA and nephelometry have been used extensively by some groups [9,10], but the majority of publications report the use of one of two commercial ELISA kits manufactured by Biovendor (Modrice, Czech Republic) and Epitope Diagnostics Inc (San Diego, USA). We compared the analytical performance of these two assays, looking at patient and normal plasma and examined the effects of glycosylation of the protein, with reference to potential biological effects.

## 2. Materials and methods

### 2.1. Study participants

We used 178 lithium heparin plasma samples collected at the baseline visit of patients enrolled in a prospective cohort study of cardiovascular risk in patients with CKD stages 3 and 4. The details of this study have been previously described [5]. In order to evaluate the effect of CKD status, samples were also obtained from a cohort of 78 otherwise unselected patients (50 male; mean age 58 ± 10.4 yr) in

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whom there was no history of type 2 diabetes mellitus nor biochemical evidence of renal dysfunction (eGFR>60 mL/min/1.73 $m^2$ and urinary protein:creatinine ratio <30 mg/mmol).

### 2.2. Plasma samples

Venous blood was collected into lithium heparin-containing Vacuette (Greiner) tubes and centrifuged at 3000 g for 15 min. Plasma samples were stored at −70 °C until assay and were not subject to repeated freeze-thaw cycles. Prior to batched-analysis, samples were allowed to thaw completely and mixed thoroughly. We prepared 3 plasma pools at low, intermediate and high concentration for use as in-house internal quality control material and for the assessment of imprecision. Concentrations were assigned by calculating the mean value of 30 replicates using the Epitope Diagnostics ELISA kit. All plasma fetuin-A measurements were made in duplicate and the mean used in subsequent analysis.

### 2.3. Fetuin-A ELISA assays

Human fetuin-A ELISA kits were obtained from Biovendor (Modrice, Czech Republic) and Epitope Diagnostics, Inc (San Diego, USA). Both assays employ a two-site 'sandwich' format utilising two polyclonal goat anti-human fetuin-A antibodies that recognise different epitopes of human fetuin-A. In each instance the manufacturer's protocol was followed exactly. Plasma samples were diluted 1/10,000 for the Biovendor assay and 1/30,000 for the Epitope Diagnostics assay to give concentrations in the 10–100 ng/mL range. In order to maximise reproducibility, the assay was programmed onto a fully-automated ELISA workstation (Triturus, Grifols, UK). The Biovendor kit uses a highly purified plasma based formulation of human fetuin-A as standard supplied as a single lyophilised stock from which other calibrators were prepared. In contrast, the Epitope Diagnostics kit supplied human fetuin-A in liquid bovine serum-based matrix. According to the product literature, values for the Epitope Diagnostics kit were assigned using recombinant fetuin-A. We performed Western blotting (using capture antibody obtained from each manufacturer) in parallel with analysis by ELISA to exclude cross-reactivity with other plasma proteins or fragments in 50 samples.

### 2.4. Assessment of analytical characteristics

We defined the limit of detection as the concentration of analyte (measured 20 times in a single batch) that generates a signal 3 standard deviations above the mean for an analyte-free matrix (assay buffer). Imprecision was evaluated by measuring 10 replicates of pooled plasma samples at 3 different concentrations in five independent batches. To assess recovery, we spiked 10 individual pre-diluted plasma samples (encompassing a range of endogenous concentrations) with low, intermediate and high concentrations of highly purified human plasma fetuin-A standard (ProSpec — Tany TechnoGene Ltd, Rehovot, Israel). Recovery was also assessed using a recombinant human fetuin-A standard, purified from a mouse myeloma cell line (RnD Systems, Abingdon, UK) and serially diluted in each kit diluent. Cross-reactivity was determined by spiking assay buffer with recombinant human fetuin-B (RnD Systems, Abingdon, UK) and bovine fetuin-A (Sigma-Aldrich, Dorset, UK) at high concentration. We observed no significant between-lot bias or variation in imprecision.

### 2.5. Glycosylation studies

We investigated the effect of fetuin-A glycosylation status on antibody specificity by randomly selecting 15 plasma samples from each cohort and subjecting them to non-denaturing enzymatic deglycosylation using a commercially available kit (Prozyme, San Leandro, USA). Reactions were monitored using SDS-PAGE and purified fetuin-A standard was run with each separation (Biovendor, Czech Republic). Complete deglycosylation was achieved after a final incubation of 24 h at 37 °C. Spiking diluent with enzyme cocktail had no effect on blank measurements in either assay. Deglycosylated samples were subsequently assayed using each ELISA kit.

### 2.6. Statistics

Deming regression and difference plots were generated using the Analyse-IT method evaluation edition add-in (Leeds, UK) for Microsoft Excel. USA). Data are expressed as the mean ± SD. Two-tailed $P<0.05$ was considered significant.

## 3. Results

The analytical characteristics of each ELISA kit are summarised in Table 1. We evaluated the agreement between results in two cohorts: firstly in 178 patients with stage 3 and 4 CKD (Fig. 1A and C) and secondly in a group of 78 patients without biochemical evidence of renal dysfunction or history of type 2 diabetes mellitus but whom were otherwise unselected (Fig. 1B and D). The mean plasma fetuin-A concentration in CKD cohort (Biovendor, 0.236 ± 0.076 g/L; Epitope Diagnostics, 0.526 ± 0.147 g/L) was significantly lower than that in the group with normal renal function (Biovendor, 0.303 ± 0.060 g/L; Epitope Diagnostics, 0.633 ± 0.115 g/L), regardless of the method used ($P<0.0001$).

Deming regression in the CKD cohort yielded slope 2.52 (95% CI 2.19–2.85), $y$-intercept −0.05 (95% CI −0.04–0.07), $S_{y|x}=0.099$ g/L, $R^2=0.694$. Regression analysis in the cohort with normal renal function yielded slope 2.05 (95% CI 1.78–2.31), $y$-intercept −0.04 (95% CI −0.12–0.04), $S_{y|x}=0.064$ g/L, $R^2=0.748$. The slopes of these two regression lines were found to be significantly different ($F$-test, $P<0.001$). In both cohorts, difference plots showed that the Epitope diagnostic kit had significant positive bias with respect to mean fetuin-A concentration: 76.4 % (95% CI 73.6–79.2) for the CKD and 61.8 % (95% CI 59.6–63.9) in the non-CKD samples.

**Table 1**
Fetuin-A ELISA analytical characteristics.

| Characteristic | Epitope Diagnostics ELISA | Biovendor ELISA |
|---|---|---|
| Limit of detection | 3.5 ng/mL | 0.4 ng/mL |
| *Within-batch imprecision (CV), %* | | |
| Low (15 ng/mL) | 4.8 | 3.2 |
| Intermediate (30 ng/mL) | 5.2 | 3.8 |
| High (90 ng/mL) | 5.4 | 3.9 |
| *Total imprecision (CV), %* | | |
| Low (15 ng/mL) | 8.1 | 5.8 |
| Intermediate (30 ng/mL) | 7.5 | 5.7 |
| High (90 ng/mL) | 7.9 | 6.1 |
| *Recovery of native plasma fetuin-A, %*[a] | | |
| Low (15 ng/mL) | 126.5 ± 3.7 | 98.3 ± 1.3 |
| Intermediate (30 ng/mL) | 123.4 ± 4.8 | 97.1 ± 2.0 |
| High (90 ng/mL) | 123.9 ± 4.1 | 101.4 ± 2.9 |
| *Recovery of recombinant fetuin-A, %*[a] | | |
| Low (15 ng/mL) | 325.1 ± 5.8 | 53.8 ± 4.2 |
| Intermediate (30 ng/mL) | 342.9 ± 6.8 | 56.4 ± 3.7 |
| High (90 ng/mL) | 375.6 ± 6.2 | 58.0 ± 4.0 |
| *Cross-reactivity* | | |
| Bovine fetuin-A (100 ng/mL) | <3.5 ng/mL | <0.4 ng/mL |
| Human fetuin-B[b] (100 ng/mL) | <3.5 ng/mL | <0.4 ng/mL |

[a] Mean ± SD.
[b] Recombinant protein.

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