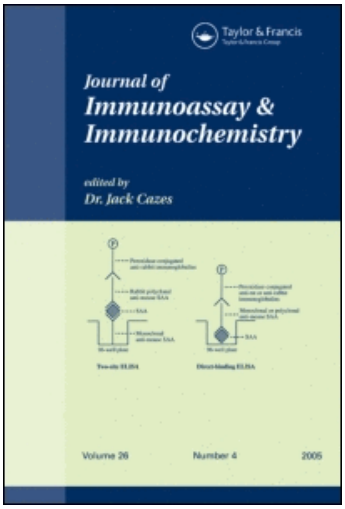

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DEVELOPMENT AND VALIDATION OF AN IMMUNOLUMINOMETRIC ASSAY (ILMA) FOR FETUIN-A-GLYCOPROTEIN

Amelie Bäumer,[1] Heinrich Schmidt-Gayk,[2,3] Michael Aulmann,[3] and Ti-Sun Kim[1]

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□ *Background: To identify dialysis patients with low fetuin-A levels, a sensitive immunoluminometric assay (ILMA) was developed. Methods: For the two-site ILMA, one monoclonal antibody was coated to polystyrene beads and one polyclonal antibody was labelled with acridinium ester. Results: The lower detection threshold was 0.013 g/L, with the normal range 0.20–0.87 g/L (arithmetic mean 0.437 ± 0.118 g/L). Serum fetuin-A levels in the dialysis patients were significantly lower (arithmetic mean: 0.352 ± 0.099 g/L, $p < 0.0001$). Conclusion: This ILMA has been proved to be a reliable method for the determination of serum fetuin-A concentrations in dialysis patients.*

Keywords cardiovascular diseases, dialysis patients, fetuin-A/α_2-Heremans-Schmid-glycoprotein, ILMA, Vascular calcification

INTRODUCTION

Fetuin-A, also known as α_2-Heremans-Schmid-glycoprotein or AHSG, is the most important inhibitor for unwanted calcification.[1] It is a multifunctional, negative acute-phase-protein,[2] which is able to inhibit the calcium phosphate-precipitation in serum[3,4] by forming a fetuin-mineral complex (FMC).[5,6] According to this model, decreased fetuin-A concentrations in serum are connected with inflammation caused, for example, by trauma or infection[7–9] and were found in patients with acute myocardial infarction (AMI),[10] trauma,[11–13] malnutrition, atherosclerosis, Pseudoxanthoma

Prof. Dr. Schmidt-Gayk passed away in September 2007.

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elasticum (PXE), metastasis spreading bone cancer, and chronic-kidney disease.[9,14–17]

Low fetuin-A levels are among the main factors for strong calcification in patients with chronic kidney disease.[8,18–20] Reduced fetuin-A levels lead to an accelerated atherosclerosis[8,21,22] and, in the end, to a higher cardiovascular and all-cause mortality.[17,23–26] Therefore, a lack of fetuin-A is a highly significant mortality factor in patients with chronic kidney disease (CKD).[27] For quantification of fetuin-A in blood samples, commercially available ELISA kits are, at present, considered to be the most efficient and accurate technique for clinical routine diagnostic.

However, their accuracy may be limited, especially when high dilution factors are necessary.[28] Several commercially available kits for quantification of fetuin-A require a predilution of the samples in a magnitude of 1:5000,[29] which may cause imprecise measurements. Thus, several authors recommend a fully automatic predilution of serum samples.[29] Nevertheless, this strategy is impractical and too difficult to handle for clinical routine measurements.

Especially patients on hemodialysis suffer from a secondary hyperparathyreoidism that can be diagnosed by high parathormone (PTH) concentration, high phosphate, and reduced calcium in serum.[30] To reduce the negative sequelae of a permanently increase PTH-concentration, these patients receive a substitution of active vitamin D (1,25-OH Vitamin D).[31] This substitution leads to an improved enteral resorption of calcium and phosphate, and, therefore, limits the production and secretion of PTH. Without the substitution of active vitamin D, these patients would develop an osteodystrophy.[32] Thus, one possible reason for the vascular artherosclerosis of dialysis patients could be seen in an increase of serum calcium after therapy with active 1,25-OH Vitamin D, while fetuin-A is reduced at the same time by the stress of dialysis as a kind of acute-phase-protein.[33] Therefore, quantification of fetuin-A seems to be of high importance in patients on hemodialysis to improve therapeutic concepts against vascular artherosclerosis and reduce their mortality.

Therefore, the aim of this study was to develop a new two-site immunoluminometric assay (ILMA) by using one monoclonal antibody and one polyclonal antibody for quantitative analysis of fetuin-A levels in blood serum.

EXPERIMENTAL

Reagents

Assay Buffers

Most chemicals were purchased from Merck AG, Darmstadt, Germany. The basic buffer PPNE 2% BSA was used for preparation of the assay

components and in the assay. One liter of assay buffer consisted of 9.49 g Na_2HPO_3, 1.84 g KH_2PO_3, 1.0 g sodium azide, 0.4 g $Na_2EDTA_2H_2O$ (Serva AG, Heidelberg, Germany), 20.0 g bovine serum albumin (Sigma GmbH, Deisenhof, Germany) and distilled water. Coating buffer (pH = 9.5) exert for tracer production and for coating polystyrene beads were made of 1.33 g Na_2CO_3, 3.15 g $NaHCO_3$, and 1 liter distilled water. For the automatic luminescence analyser, we used Magic Lite Analyser (Reagents 1 + 2) (Ciba Corning Diagnostik GmbH, Fernwald, Germany). To brand the tracer-concentrate, an acridinium labelling kit was used (Bio Trend Chemikalien GmbH, Köln, Germany).

Antibodies

During the assay development, we used five monoclonal antibodies (MAHS-2, MAHS-3, MAHS-4, MAHS-7, and a mixture of MAHS-3 + 7 purchased from the University of Aachen, Germany) in a concentration of 20 ng/tube and 40 ng/tube and three polyclonal antibodies (sheep anti human α_2HS-glycoprotein, Binding Side, Heidelberg, Germany; AS 5359 IgG (pH = 2.3 Eluat), University of Aachen, German; anti-fetuin-A polyclonal antibody, Epitope Diagnostics, San Diego, USA). The polyclonal antibody AS 5359 IgG (pH = 2.3 Eluat), a rabbit anti-human IgG polyclonal antibody specific for fetuin-A, and the monoclonal antibody MAHS-3 mixed with MAHS-7 in a ratio of 1:1.

Coating Procedure

After attenuation of the polyclonal antibody AS 5359 in a coating buffer solution, the antibody solution was lavished over approximately 8000 polystyrene beads (Spherotech GmbH, Fulda, Germany) and then incubated for 48 h at 4°C to build the solid phase. After washing with distilled water for three times, the beads were blocked with assay buffer and kept at 4°C. The beads were applicable for a period of up to 6 months.

Labelling Procedure

The purified monoclonal antibodies were branded with 20 µL of the acridinium labelling kit. After an incubation of 30 minutes in the dark and at room temperature, 20 µL lysine solution (10 g lysine in 100 mL bicarbonate buffer) was given into the mixture to stop the binding between antibody and acridinium ester; further 15 minutes incubation followed. A separation of labelled antibodies from free acridinium ester was achieved using a centrifugal varifuge 3.0 (Heraeus Sepatech, Hanau, Germany) (3200 rpm, 300 µg). The purified labelled antibody was diluted with the assay buffer to a concentration of 40 ng per tube. Additionally, 1% normal-mice-serum and 1% normal-rabbit-serum were added to minimize a potential HAMA-effect, after which the tracer was stored in aliquots at −20°C.

Standards and Controls

The standards were made of the standards SL BZ (University Aachen, Germany) and prepared from blood serum samples with known fetuin-A concentrations and diluted in assay buffer (PPNE 2% BSA) to the particular standard concentration of 0.0 mg/L (pure assay buffer), 0.156 mg/L, 0.625 mg/L, 2.5 mg/L, 10.0 mg/L, and 40 mg/L.

The standards with 2.5 mg/L and 10.0 mg/L, which were in the measurement range of the patients probes, served as controls.

Patient Samples

392 serum samples were collected in the Laboratory Limbach (Heidelberg, Germany). Sera from 127 healthy, 22- to 65-years old men with an PSA-value under 2.5 μg/L and 128 healthy, not pregnant (β-HCG < 0.3 IU/L) women at the age of 9 to 66 years presented the control group. Also, 137 blood samples of renal dialysis patients were randomly chosen.

Assay Protocol

The immunoluminometric assay was performed in two incubation steps: first 10 μL of standards, controls or patient samples and 250 μL buffer were pipetted into the test-tubes (4 mL, W. Sarstedt, Nürnbrecht-Rommelsdorf, Germany) fitting the tube racks (Berthold 50-tube racks, Berthold, Germany). Then one coated bead was added to each tube and an incubation at room temperature followed. After 2 hours, each tube was washed with 20 mL distilled water in the immuno washer LB 9200 (Berthold, Germany). 250 μL tracer were given into each tube and incubated at room temperature for 4 hours. A second washing step was done to remove unbound antibodies. Finally, chemiluminescence activity was measured in the Luminometer LB 954 (Berthold, Germany).

Statistical Analysis

To characterize the variation in repeated measurements, standard descriptive statistical parameters were calculated (arithmetic mean, median, standard deviation, coefficient of variation, 95% CI for mean, and median). Distributions of fetuin-A concentrations in samples of dialysis patients and healty controls were tested for normality with a Kolmogorov-Smirnov-test. Since the concentrations in the subgroup of healthy women did not show a normal distribution, the following statistical analyses were done with a non-parametric test exclusively (Mann-Whitney-U-Test).

Age-related changes of fetuin-A levels were sought by using ANCOVA, adjusting for gender. Statistical analysis was performed using a PC program (Systat^{TM} for Windows Version 16.0, Systat Inc., Evanston, IL, USA).

RESULTS

Analytical Evaluation

First, we tested 16 different combinations of monoclonal and polyclonal antibodies to find the one with the highest RLU-results. We selected the combination of MAHS-3 + 7 (40 ng/tube) and AS 5349 because of the wide margin from 25.987 RLUs to 950.487 RLUs.

Assay incubation was performed at 4°C and at room temperature for 1, 2, 4, and 24 h. Because of the high luminous efficiency, the assay time could be reduced in comparison to the usual assay time from 20 h to 6 h, so that the results are available on the same day. The standard curve for fetuin-A is shown in Figure 1.

Intra-assay and inter-assay variations were evaluated with three and four patient samples, respectively, at low, medium, and high fetuin-A concentrations. Intra-assay coefficients of variation (CV) were 7.7%, 5.3%, 6.3%, and 3.7%; inter-assay CVs 6.32%, 10.0%, and 13.27% (Table 1).

The compound precision profile for the assay standards with concentrations being in human serum showed CVs of 5.7% to 14.25%, standard 1 and 5 revealed higher but acceptable variances with CVs of 25.3% and 20.6%.

For the determination of the analytical detection limit, the zero standard containing pure assay buffer was measured 24 times. The mean of these results plus 3 S.D.s showed a detection limit of 10.3 mg/L.

The linearity of the assay was tested with serial dilution of three serum samples with the assay buffer. Comparison between the measured fetuin-A values and the theoretical fetuin-A values presented a good correlation.

To detect the recovery, six serum samples were mixed with standards and measured. The recovery was 73–113%, the median 93% (Table 2).

Comparing frozen samples (−20°C) with samples kept at room temperature (22°C), at 2–8°C, and at 37°C over three days, stability studies

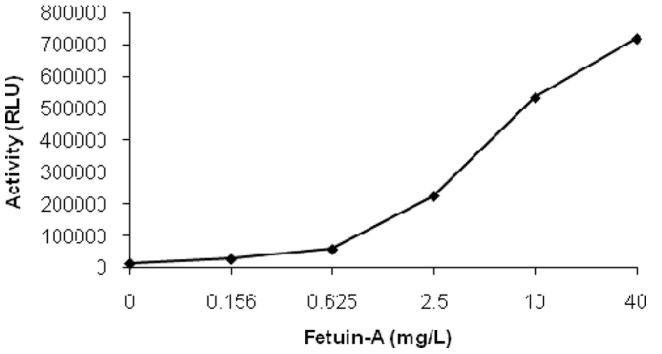

FIGURE 1 Standard curve of the assay for fetuin-A after reduction of first and second incubation time.

TABLE 1 Precision of the New ILMA for Quantification of fetuin-A

	n	mean (g/L)	S.D. (g/L)	CV (%)
Intraassay				
Serum 1	12	0.27	0.02	7.22
Serum 2	12	0.34	0.02	5.27
Serum 3	12	0.48	0.03	6.26
Interassay				
Serum 1	12	0.19	0.012	6.32
Serum 2	12	0.36	0.036	10.00
Serum 3	12	0.52	0.069	13.27

Different control samples were used for the determination of intra-/interassay variations.

pointed only a mean deviation. Similar results were observed by the storage of standards. The temperature had no significant effect on the stability in this case. Only the RLU-values declined by keeping the tracer at room temperature over three days compared to tracer saved at −20°C and at 2–8°C. Multiple frozen standards showed marginal variances; the tracer demeaned similar. Samples with higher concentrations (>0.30 g/L) showed more fluctuation than samples with lower concentrations.

TABLE 2 Analytical Recovery of Standards Added to Human Sera (1:1)

Sample No.	Fetuin-A Conc. (g/L)	Added (g/L)	Found (g/L)	Recovery (%)
Sample 1	0.26	0.016	0.140	101.5
Sample 1	0.26	0.063	0.162	85.8
Sample 1	0.26	0.250	0.270	105.8
Sample 1	0.26	1.000	0.544	86.4
Sample 2	0.16	0.016	0.107	121.6
Sample 2	0.16	0.063	0.127	113.4
Sample 2	0.16	0.250	0.197	96.1
Sample 2	0.16	1.000	0.495	85.4
Sample 3	0.37	0.016	0.178	92.2
Sample 3	0.37	0.063	0.213	98.2
Sample 3	0.37	0.250	0.255	82.3
Sample 3	0.37	1.000	0.599	87.5
Sample 4	0.32	0.016	0.121	72.0
Sample 4	0.32	0.063	0.203	105.7
Sample 4	0.32	0.250	0.256	89.7
Sample 4	0.32	1.000	0.575	87.1
Sample 5	0.57	0.016	0.259	88.4
Sample 5	0.57	0.063	0.315	96.9
Sample 5	0.57	0.250	0.418	102.0
Sample 5	0.57	1.000	0.671	85.5
Sample 6	0.57	0.016	0.234	79.9
Sample 6	0.57	0.063	0.234	73.8
Sample 6	0.57	0.250	0.359	87.6
Sample 6	0.57	1.000	0.831	105.1

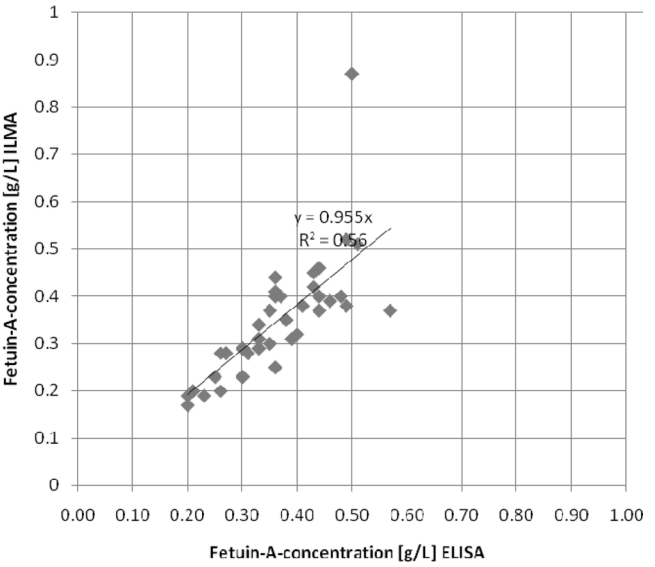

FIGURE 2 Correlation between ELISA and ILMA (regression analysis).

The comparison with a commercially available ELISA (Epitope Diagnostics, San Diego, CA, USA) that has been used previously in our laboratory yielded comparable concentrations, however, with remarkable deviations in particular cases (Fig. 2). The ELISA tended to measure slightly higher fetuin-A concentrations compared to the ILMA for the majority of samples (25 out of 38, =65.7%) under analysis. However, for most patients

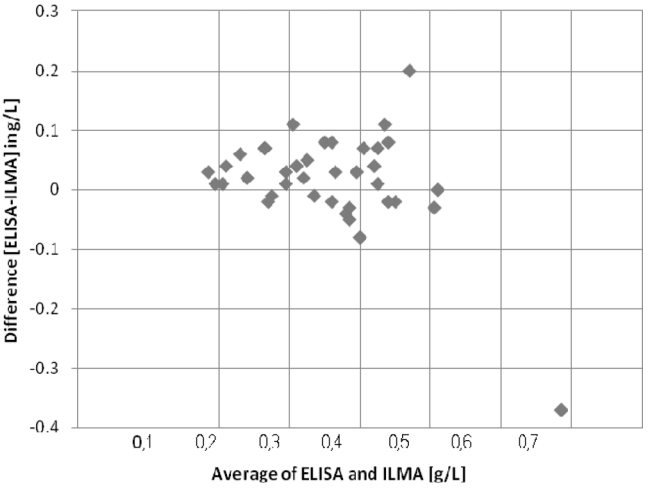

FIGURE 3 Differences between ELISA and ILMA plotted against average concentration.

TABLE 3 Passing Bablok Regression Analysis

Variable X	ILMA
Variable Y	ELISA
Sample size	38

	Variable X	Variable Y
Lowest value	0,1700	0,2000
Highest value	0,8700	0,5700
Arithmetic mean	0,3468	0,3645
Median	0,3450	0,3600
Standard deviation	0,1261	0,09374
Standard error of the mean	0,02045	0,01521

Regression Equation	
$y = 0{,}04542 + 0{,}9167x$	
Intercept A	0,04542
95% CI	−0,01786 to 0,1000
Slope B	0,9167
95% CI	0,7500 to 1,1429
Cusum test for linearity	No significant deviation from linearity ($P > 0.10$)

(89.5%), the difference between ELISA and ILMA did not exceed a range of ±0.10 g/L (Fig. 3). With a Passing Bablok regression analysis, the following regression equation was calculated: $y = 0{,}04542 + 0{,}9167x$ (Table 3).

Clinical Results

The normal range of fetuin-A was detected on the basis of 288 serum samples from healthy subjects, which were sent to the Laboratory Limbach,

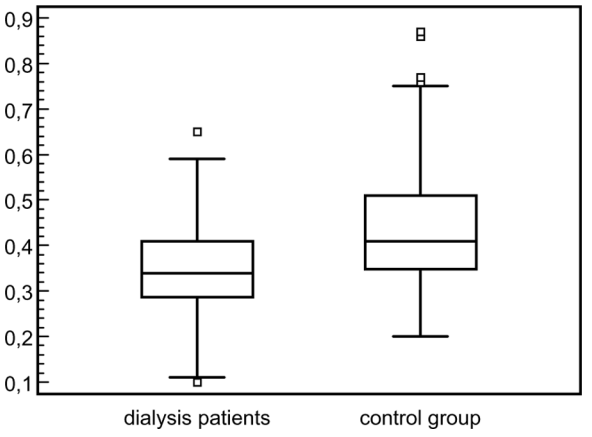

FIGURE 4 Concentration of fetuin-A (g/L) in sera from CKD-patients and in the control group.

TABLE 4 ANCOVA of Fetuin-A Concentrations by Age with Gender as Covariant (GLM)

Source	Type III Sum of Squares	Df	Mean square	F	Significance
Corrected Model	1.044[a]	54	0.019	1.546	0.017
Intercept	13.125	1	13.125	1050.216	0.000
Gender	0.011	1	0.011	0.897	0.345
Age	0.980	53	0.018	1.480	0.029
Error	2.487	199	0.012		
Total	52.100	254			
Corrected Total	3.530	253			

[a]R Squared = 0.296 (Adjusted R Squared = 0.104).

Heidelberg, Germany, in the context of a routine check-up. 128 samples from non- pregnant women (β-HCG <0.31U/L) were quantified and showed a mean of 0.45 ± 0.14 g/L, 127 were male's samples (PSA-value <2.5 µg/L) with a mean of 0.42 ± 0.088 g/L. As the data in the subgroup of the female controls showed no normal distribution, comparison between female and male values was calculated with a non-parametric test (Mann-Whitney-U-Test). Differences between male and female control subjects were not statistically significant ($p > 0.05$).

Furthermore, 137 serum samples from dialysis patients were measured. The arithmetic mean in this group was 0.352 ± 0.099 g/L, the median 0.34 (range: 0.10–0.65 g/L). In male patients, the average serum concentration of fetuin-A was 0.342 ± 0.102 g/L) (female patients: 0.363 ± 0.093 g/L). The difference between male and female showed no statistically significant difference ($p > 0.05$).

The difference of arithmetic means between dialysis patients and healthy controls was statistically significant, both for the total number of subject and the separate analyses for male and female subjects for ($p < 0.0001$, Mann-Whitney-U-test) (Fig. 4).

For healthy subjects, ANCOVA analysis showed that age had a statistically significant correlation on fetuin-A-levels ($p < 0.05$), while gender was not a significant covariate (Table 4). For patients under hemodialysis, no significant correlation between fetuin-A-levels and age could be demonstrated ($p > 0.05$).

DISCUSSION

Fetuin (α_2-HSG, or fetuin-A) is a glycoprotein present in the circulation, synthesized by hepatocytes. The fetuin molecule consists of two polypeptide chains, which are both cleaved from a proprotein encoded from a single mRNA. The protein is commonly present in the cortical plate of the immature cerebral cortex and bone marrow hemopoietic matrix. However,

its function of inhibiting soft tissue calcification is achieved by forming a soluble colloidal microsphere of fetuin-calcium-phosphate complex in the blood stream. Fetuin-A is known to have the highest capacity in inhibiting soft tissue calcification among all other molecules in the circulation.[17,23–26]

It has been reported, several times, that decreased fetuin-A-levels are significantly associated with increased vascular calcification[8,18–20] and that vascular calcifications based on a lack of fetuin-A are causing a greater cardiovascular and all-cause mortality.[9,17,23,24,26] This association could be found in patients with CKD on long- and short-term haemodialysis,[34] making a lack of fetuin-A a highly significant mortality factor in patients on dialysis.[27] Thus, a periodic observation of serum fetuin-A-levels in this patient group is indicated.

Fetuin-A addition in vitro increases the ability of binding calcium phosphate in serum, so the synthetic production of fetuin-A may offer a new possibility in preventive therapy and treatment of dialysis patients.[18,35] For measuring the serum fetuin-A levels, an immunoluminometric assay (ILMA) was developed as an alternative to the widely used Human Fetuin-A ELISA kit (Epitope Diagnostics, San Diego, CA).

This ELISA may lead to inaccurate results, especially if a high dilution of the original sample is necessary.[28] Nephelometry is another method to detect fetuin-A, which showed very stable and reproducible results and is discussed to be superior to the ELISA technique.[28,36]

For the new ILMA, we could identify an analytical detection limit of 0.0103 g/L, which is in the same magnitude as the detection limits of the commercially available ELISA kits for quantification of fetuin-A in blood samples. The new ILMA also exhibited sufficient linearity data and coefficients for inter- and intra-assay variation, indicating that this technique can be considered suitable for accurate measurement of serum fetuin-A under clinical routine settings. The recovery data that varied between 73–113%, depending on the concentration of the standard, proved the high precision of the assay.

The monoclonal antibody against human fetuin-A is species-specific and the polyclonal one monospecific, so it was not necessary to test its specifity. The reduction of total assay time from approximately 20 h to 6 h now allows to have final test results on the day of receipt of samples.

The normal range of the new assay, based on 255 serum samples, was 0.2–0.87 g/L, with an arithmetic mean of 0.437 ± 0.117 g/L. In contrast to Dickson et al.[37] we could not show a gender specific decrease of fetuin-A values with age for women. With a GLM/ANCOVA-procedure, we could identify an age-dependent decrease of fetuin-A for the healthy controls, but could not prove this effect for a specific gender statistically. Jahnen-Dechent et al.[1] assumed that there may be a relationship between osteoporosis and a possible decrease of serum fetuin-A levels in older

women. We could not corroborate the thesis by Dickson et al.[37] that men would have higher fetuin-A values than women of the same age. In comparison to serum fetuin-A values given in the literature (Ketteler et al.[34]: 0.5 to 1.0 g/L, Wang et al.[9]: 0.4 to 0.95 g/L), the reference values for healthy controls were lower for our ILMA. This was also the case for the fetuin-A-values of our dialysis patients (0.10 to 0.65 g/L, mean 0.35 g/L). Thus, the difference between the means of control group and patient group of 0.09 g/L for our new ILMA was exactly located within the range given in literature (Ketteler et al.[34] : 0.06 g/L, Mazzaferro et al.[38] : 0.13 g/L). These data also are in agreement with the fact that we found slightly higher fetuin-A concentrations with the reference ELISA in the majority (65.7%) of the samples compared to the ILMA results.

Wang et al.[9] showed that an increase of 0.01 g/L in serum fetuin-A-levels is associated with a 6% risk-decrease to get a valvular calcification. Refer to that our result would mean that dialysis patients had a risk-increase of 54% to get a valvular calcification, which is reflected in a 10 to 30 times higher cardiovascular mortality in this patients.[39–41] If differences in dialysis-calcium levels, a high calcium supply or active vitamin D dose or rather calcium phosphate products have influence on the fetuin-A levels in serum has not been investigated until now. Hermans et al.[36] found that an increase in the serum fetuin-A concentration of 0.1 g/L was associated with a significant reduction in all-cause mortality of 13%. There was a significant 17% reduction in non-cardiocvascular mortality and a near significant reduction in cardiovascular mortality. This association of fetuin-A and mortality rates was comparable in both hemodialysis and peritoneal dialysis patients, even when corrected for factors including, but not limited to age, gender, primary kidney disease, C-reactive protein levels, and nutritional status. They concluded that serum fetuin-A concentrations may be a general predictor of mortality in dialysis patients. Thus, measurement of fetuin-A seems to be of high importance in patients on hemodialysis to improve therapeutic concepts against vascular artherosclerosis and reduce their mortality.

CONCLUSIONS

We developed a new two-site immunoluminometric assay for fetuin-A with one monoclonal and one polyclonal antibody. It is a reliable and simple method and detects fetuin-A with a detection limit of 0.013 g/L. The assay exhibits a normal range of 0.20–0.87 g/L. The new ILMA shows a high correlation with the already established ELISA kits for fetuin-A quantification that are commercially available. The new ILMA may contribute to the identification of dialysis patients with a high risk of vascular calcification, who should be managed aggressively.[36]

When fetuin-A concentrations from studies using ELISA techniques are compared with results obtained by our new ILMA technique, it should be kept in mind that ILMA concentrations tend to be lower in a range between 0–0.1 g/L in approximately two thirds of all cases.

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