Novel diagnostics and therapeutics to prevent injury in native and transplanted kidneys

Groeneweg, K.E.

Chapter 2

Serum TIMP-2, but not IGFBP7, levels remain high despite successful simultaneous pancreas-kidney transplantation

Koen E. Groeneweg, Fred P.H.T.M. Romijn, Roel Bijkerk, Cees van Kooten, Marlies E.J. Reinders, Christa M. Cobbaert, Johan W. de Fijter

Submitted

Abstract

Background

Insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases 2 (TIMP-2), both involved in the G1 cell cycle arrest, have mainly been described as urinary biomarkers in the context of acute kidney injury. Elevated serum levels, however, have also been reported in patients with diabetes mellitus (DM) with impaired kidney function. Differentiation between kidney injury and systemic vascular damage may be difficult, especially in the early stages of diabetic nephropathy. The objective of this study was to assess urinary and serum IGFBP7 and TIMP-2 levels in type-1 diabetics with either preserved renal function or advanced renal dysfunction (i.e. eGFR ≤30ml/min/1.73m^2), as well as recipients of a successful kidney (KTA) or simultaneous pancreas-kidney transplant (SPKT). SPKT recipients were followed longitudinally during the first year after transplantation to clarify the course of these biomarkers in case of replacement of both kidney and endogenous pancreas function.

Methods

Serum and urinary IGFBP7 and TIMP-2 concentrations were measured using ELISA assays, in 96 individuals; patients with type-1 DM with an eGFR >30ml/min/1.73m^2 (DM>30; n=13), DM patients with an eGFR ≤30ml/min/1.73m^2 (DM≤30; n=17), healthy controls (HC; n=14), and recipients of a KTA (n=14) or SPKT (n=36). DM≤30 patients who received a SPKT (n=18) were followed at 1, 6 and 12 months after transplantation.

Results

Circulating IGFBP7 and TIMP-2 were significantly higher in DM≤30 as compared to HC. In addition, both circulating IGFBP7 and TIMP-2 decreased rapidly after a successful SPKT and IGFBP7 remained low during follow-up. The serum IGFBP7 level was highly dependent upon glomerular filtration. In contrast, despite adequate kidney graft function, circulating TIMP-2 levels returned within the first year to levels comparable to those found in patients with DM>30 or after KTA. In addition, TIMP-2 correlated significantly with the vascular injury marker angiopoietin-2. Urinary levels of either IGFBP7 or TIMP-2 showed a high variation, but did not differ significantly between the different groups.

Conclusions

Circulating IGFBP7 and TIMP-2 levels were higher in type-1 diabetics with impaired renal function. While increased IGFBP7 levels were associated with glomerular filtration, TIMP-2 levels remained significantly higher in type-1 diabetics after a successful SPKT, suggesting persistent and chronic vascular injury.

Introduction

Insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases 2 (TIMP-2) are both biomarkers involved in G1 cell cycle arrest and initially described as urinary biomarkers in the context of acute kidney injury.[1-3] Because of the reported predictive value of IGFBP7 and TIMP-2 for clinical acute kidney injury prediction,[4,5] the U.S. Food and Drug Administration approved these markers for this purpose. Next to acute kidney injury, increased IGFBP7 and/or TIMP-2 levels have been reported in the context of systemic (vascular) diseases, such as diabetes mellitus (DM),[6,7] chronic kidney injury and chronic allograft injury after renal transplantation.[8-10] The assumption of a role in the pathophysiology of vascular disease is further supported by the association of IGFBP7 and TIMP-2 with non-renal diseases, such as malignancies[11,12] and endometriosis.[13] The association between IGFBP7 and TIMP-2 is also of interest in relation to microvascular complications and evolution of diabetic nephropathy (DN). The prognosis of type-1 diabetes is highly dependent on the progression of microvascular complications, such as neuropathy, retinopathy and nephropathy. Diabetic nephropathy is a common vascular complication in type-1 diabetes[14] and may lead to end-stage renal disease in a relevant proportion of patients. The primary goal of treatment in patients with diabetic nephropathy is preservation of kidney function by optimal glycemic control and reduction of albuminuria by adequate blood pressure regulation, in order to prevent or at least delay the need for renal replacement therapy.[15] Progression to end-stage renal disease is however diverse and difficult to predict for individual patients with diabetic nephropathy. More knowledge is needed about the pathophysiology to clarify this phenomenon and biomarkers are of great importance to identify patients in an early stage of DN.

In case of progression to end-stage renal disease, a simultaneous pancreas kidney transplant (SPKT) is the preferred treatment option that replaces kidney function and also restores endogenous insulin secretion. Changes over time of serum TIMP-2 and IGFBP7 levels after SPKT and a kidney transplant alone (KTA) are unclear, but may provide relevant information on the etiology of the altered IGFBP7 and TIMP-2 levels observed in diabetic nephropathy and potentially differentiate between a change in glomerular filtration and/or ongoing systemic (micro)vascular disease. The aim of this study was to assess urinary and serum IGFBP7 and TIMP-2 levels in the context of type-1 diabetes with and without end-stage renal disease. Subsequently, changes were studied in type-1 DM patients after (pancreas) kidney transplantation.

Materials and methods

Study cohort

This single-center, cross-sectional study consists of 96 individuals enrolled at the Leiden University Medical Center (LUMC) with a one-year follow-up in the subgroup who received a combined pancreas-kidney transplant. The cross-sectional cohort consisted of five groups; patients with type-1 DM with preserved renal function (estimated glomerular filtration rate (eGFR) >30ml/min/1.73m^2; DM>30: n=13), type-1 DM patients with impaired renal function (eGFR ≤30ml/min/1.73m^2; DM≤30: n=17), healthy controls (HC: n=14), and type-1 DM patients with end-stage renal disease who received a SPKT (n=36) or KTA (n=14). The KTA group consisted of type-1 DM patients not suitable for SPKT (n=11) or those with an early failed pancreas graft due to vascular thrombosis within four days after transplantation (n=3). The DM≤30 patients from the cross-sectional study who received an SPKT had a follow-up of 1, 6 and 12 months after transplantation. Baseline characteristics of the cross-sectional study, as well as the clinical follow up characteristics of the SPKT subgroup (body mass index, blood pressure, HbA_{1c}, glucose, eGFR, and proteinuria), were retrieved from the electronic health records. All SPKT and KTA patients were transplanted at the LUMC between 1991 and 2012 and received the immunosuppressive regime according to the protocol at the time of transplantation, as previously described.[16,17]

The study design was approved by the Medical Ethical Committee of the LUMC. Written informed consent was obtained from all participants.

Assessment of urinary and serum TIMP-2 and IGFBP7 levels

Blood and urinary samples from all participants were obtained in the outpatient clinic and directly centrifuged and stored at -80°C. Urinary and serum TIMP-2 and IGFBP7 were quantified using sandwich enzyme-linked immunosorbent assays (ELISA) according to manufacturer's instructions (ELISA, Cat. Nr. DTM200, R&D systems, Minneapolis, MN for TIMP-2, and Cat. Nr. EK0991, Boster Biological Technology, Pleasanton, CA for IGFBP7, respectively). Concentrations of urinary and serum TIMP-2 and IGFBP7 were within linear range after sample dilution. Analysis for TIMP-2 and IGFBP7 was performed with one reagent lot number. Low and high-level quality control (IQC) urine samples were prepared from pooled urine by spiking and analyzed in triplicate on each sample plate to assess the stability of the assay. The mean analytical imprecision (expressed as CV%) for serum TIMP-2 was 2.8% (at 3292 pmol/l). For urinary TIMP-2, analytical imprecision was 4.1% (at 183 pmol/l) for low IQC and 4.4% (at 239 pmol/l) for high IQC. For urinary IGFBP7, CV% was 11.5% (at 831 pmol/l) for low IQC and 9.1% (at 2141 pmol/l) for high IQC. Creatinine and total protein were measured using a Cobas c502 analyzer (Roche Diagnostics, Mannheim, DE), according to the manufacturer's instructions. The vascular marker angiopoietin-2 (Ang-

2), previously determined in this cross-sectional cohort,[16,18] was included in the analysis of this study as marker for vascular injury.

Statistical analyses

Parametric data are described as mean ± SD, non-parametric data as median and IQR, and categorical data as numbers and percentages. Baseline characteristics were tested for differences using one-way ANOVA (parametric data), Kruskall-Wallis (non-parametric data), and Fisher Exact test (categorical data). Baseline characteristics of the longitudinal cohort were tested for differences using the paired T-test (parametric data), Wilcoxon signed-rank test (non-parametric data) and Friedman's two-way ANOVA by ranks (categorical data).

Urinary levels were corrected for urinary creatinine from the same sample. Circulating and urinary IGFBP7 and TIMP-2 levels were both reported as logarithmic levels and herewith showed a normal distribution. Fractional excretion of IGFBP7 and TIMP-2 were calculated by ([urinary biomarker] x [serum creatinine] / [serum biomarker] x [urinary creatinine]) and showed a normal distribution after logarithmic transformation. Cross-sectional results were analyzed with a univariate general linear model. Longitudinal results were analyzed using a linear-mixed model analysis. Correlations were analyzed using a Spearman rank correlation.

A value of $p < 0.05$ was considered as statistically significant. Data analysis was performed with SPSS version 23.0 (SPSS Inc., Chicago IL, USA) and creation of graphs with GraphPad Prism version 8.0 (Graphpad Prism Software Inc., San Diego, CA, USA).

Results

Patient characteristics

Baseline characteristics of the participants are summarized in *Table 1*. The median time since the diagnosis of type-1 DM in this cohort was 30 years (IQR 22-38 years) and comparable in patients with DM≤30 and SPKT recipients. After a successful SPKT or KTA the mean eGFR was significantly better as compared to DM≤30 patients (both <0.001). The mean HbA_{1c} was normal and significantly lower in the SPKT recipients as compared with all other subgroups of diabetics. The organs of all SPKT and 36% of KTA originated from deceased donors. All SPKT and 79% of KTA patients received a calcineurin inhibitor (tacrolimus or cyclosporin) and prednisone use was 69% and 64% in the SPKT and KTA group respectively. The type-1 DM patients in the longitudinal study had adequate glucose regulation after successful SPKT (HbA_{1c} 37.7 ± 8.7 and glucose levels 5.8 ± 2.8) and mean eGFR of their kidney was 54 ± 12 one year after transplantation (*Table 2*). In addition, these SPKT patients had a mean blood pressure of 128/77 one year after transplantation and minimal proteinuria. Patient and graft (both kidney and pancreas) survival were 100% in the study period.

Table 1. *Cross-sectional study patient characteristics.*

Characteristics	HC (n=14)	DM>30 (n=13)	KTA (n=14)	DM≤30 (n=17)	SPKT (n=36)
Sex, *male, n (%)*	7 (50%)	6 (46%)	6 (43%)	13 (77%)	23 (64%)
Age *(years)*	48 ± 11	52 ± 14	48 ± 10	45 ± 6	48 ± 8
BMI *(kg/m^2)*	25.0 ± 3.7	23.9 ± 2.5	25.2 ± 4.8	24.9 ± 3.4	24.3 ± 4.4
Systolic BP *(mmHg)*	132 ± 14	129 ± 11	136 ± 30	147 ± 18	139 ± 23
Diastolic BP *(mmHg)*	83 ± 7	72 ± 10	80 ± 14	85 ± 9	83 ± 13
Smoking, *n (%)*	0	2 (15%)	1 (7%)	0	3 (8%)
Duration of diabetes *(y)*	-	34 + 10	36 ± 9	28 ± 9	27 ± 8
Time since Tx *(months) median (IQR)*	-	-	25 (10-65)	-	45 (19-107)
HbA$_{1c}$ *(mmol/mol)*	-	57 ± 13	70 ± 10	74 ± 20	38 ± 9
Glucose *(mmol/l)*	5.3 ± 0.9	12.6 ± 4.9	13.6 ± 6.6	12.9 ± 6.6	5.8 ± 2.8
eGFR *(ml/min/1.73m^2)*	91 ± 14	72 ± 24	64 ± 23	18 ± 7	53 ± 20
Proteinuria *(g/24 h) median (IQR)*	-	0.26 (0.18-0.41)	0.21 (0.18-0.23)	0.75 (0.54-1.30)	0.28 (0.19-0.82)

HC = healthy controls, DM>30 = diabetes mellitus with an eGFR >30ml/min/1.73m^2, DM≤30 = diabetes mellitus with an eGFR ≤30 ml/min/1.73m^2, SPKT = simultaneous pancreas-kidney transplantation, KTA = kidney transplantation alone, BMI = body mass index.

Table 2. *Longitudinal study patient characteristics of type-1 diabetes patients with an eGFR ≤30 ml/min/1.73m^2, who received a simultaneous pancreas-kidney transplantation (n=18).*

Characteristics	D0 (n=14)	M1 (n=12)	M6 (n=14)	M12 (n=12)
Systolic BP *(mmHg)*	151 ± 17	132 ± 14	131 ± 20	128 ± 19
Diastolic BP *(mmHg)*	86 ± 10	78 ± 9	78 ± 10	77 ± 5
HbA$_{1c}$ *(mmol/mol)*	74 ± 18	45 ± 18	34 ± 3	36 ± 3
Glucose *(mmol/l)*	13.7 ± 6.7	6.5 ± 1.1	5.2 ± 1.2	6.1 ± 1.7
eGFR *(ml/min/1.73m^2)*	17 ± 7	48 ± 17	52 ± 13	54 ± 12
Proteinuria *(g/24 h) median (IQR)*	0.75 (0.67-1.29)	0.63 (0.27-1.05)	0.34 (0.25-0.98)	0.26 (0.12-0.50)

D0 = before transplantation, M1 = 1 month after transplantation, M6 = 6 months after transplantation, M12 = 12 months after transplantation, BP = blood pressure, BMI = body mass index.

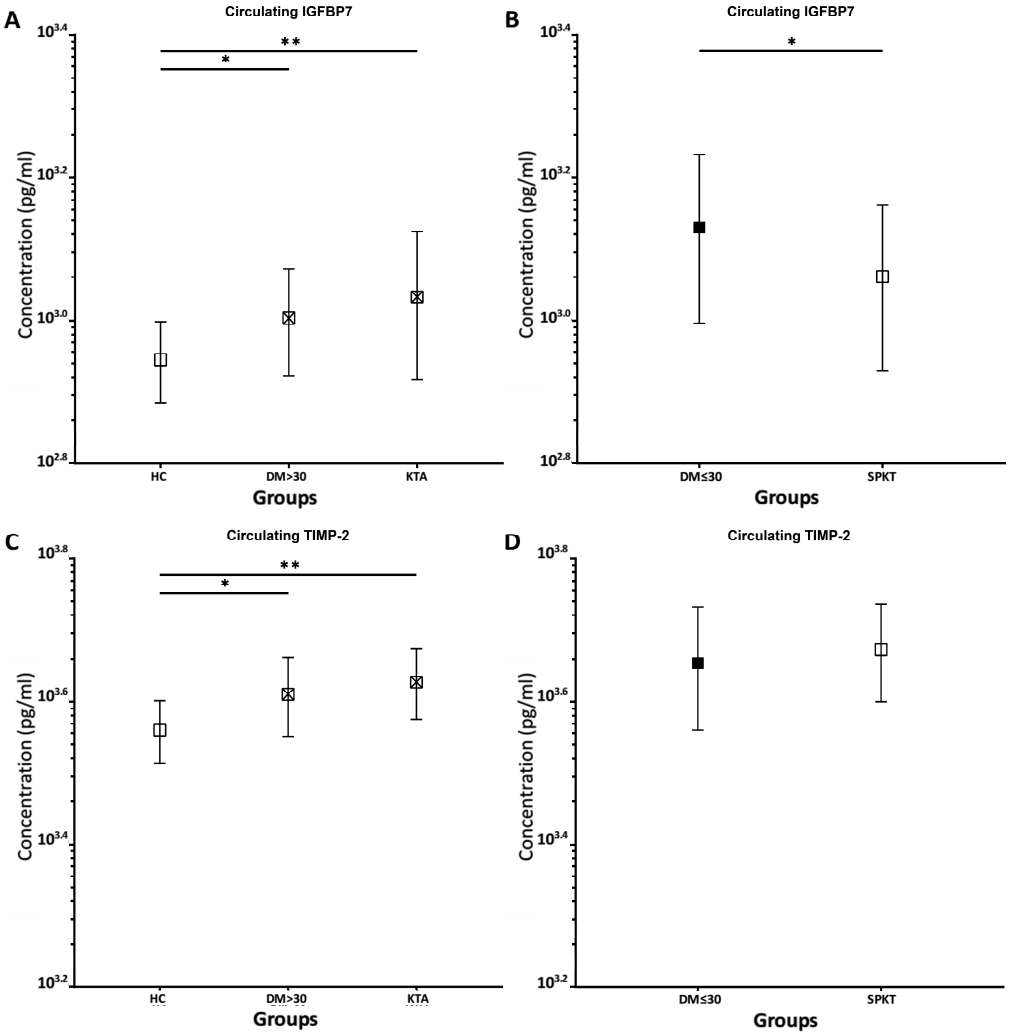

Figure 1. Circulating IGFBP7 and TIMP-2 levels are differently affected by changes in kidney function. *Circulating IGFBP7 (A) and TIMP-2 (C) levels are higher in type-1 DM patients with an eGFR >30ml/min/1.73m^2 (DM>30) or after a successful kidney transplantation alone (KTA), compared with healthy controls (HC). High IGFBP7 levels in type-1 DM patients with an eGFR ≤30ml/min/1.73m^2 (DM≤30) (B) decrease after a simultaneous pancreas-kidney transplantation (SPKT). This is in contrast with TIMP-2 levels (D) that remain high in SPKT patients. * p < 0.05, ** p < 0.01.*

Type-1 diabetes and circulating IGFBP7 and TIMP-2 concentrations

Patients with type-1 diabetes and an eGFR >30ml/min/1.73m^2 (DM>30) had significantly higher circulating IGFBP7 (p=0.04) and TIMP-2 (p=0.02) levels, as compared to healthy controls (*Figure 1*). In case of an eGFR ≤30ml/min/1.73m^2 (DM≤30), the IGFBP7 concentration was significantly higher (p=0.003) as compared to DM>30, while TIMP-2 levels were comparable (p=0.18). Type-1 diabetics, who received a successful KTA, had similar levels of IGFBP7 and TIMP-2, as found in type-1 DM patients with eGFR >30ml/min/1.73m^2. Urinary IGFBP7 and TIMP-2 levels, either corrected for urinary creatinine concentration or expressed as fractional excretion in relation to creatinine, showed a wide variation but no statistically significant differences between the different groups in this cohort with an eGFR >30ml/min/1.73m^2 (*Supplementary Figure 1*). In addition, no significant correlations between urinary IGFBP7 or TIMP-2 levels with the corresponding circulating levels were found.

Only serum IGFBP7 decreased after simultaneous pancreas-kidney transplantation

In order to further differentiate between impaired glomerular filtration and ongoing systemic vascular disease in DM patients, samples of DM≤30 patients and DM patients who received an SPKT were analyzed. Circulating IGFBP7 decreased after a successful SPKT transplant, compared with DM≤30 (p=0.03). TIMP-2, however, remained at higher levels (p=0.07), despite having adequate kidney transplant function and restored endogenous insulin secretion.

Dynamics of TIMP-2 and IGFBP7 after a simultaneous pancreas kidney transplantation

Follow-up of DM patients who received an SPKT in the first year after transplantation showed IGFBP7 to decline rapidly and remain significantly lower (p<0.001), while TIMP-2 levels, only temporarily declined after the successful SPKT (p<0.001) with a gradual return towards pre-transplant levels (*Figure 2*). Theoretically this could be explained by the gradual lowering of immunosuppressive drugs, (return of) chronic systemic inflammation, or presence of chronic vascular injury. To test this hypothesis, we evaluated the correlation of circulating IGFBP7 and TIMP-2 with the vascular biomarker Ang-2. TIMP-2 correlated significantly with Ang-2 (r=0.25, p=0.021), while IGFBP7 was not significantly correlated (r=0.15; p=0.18).

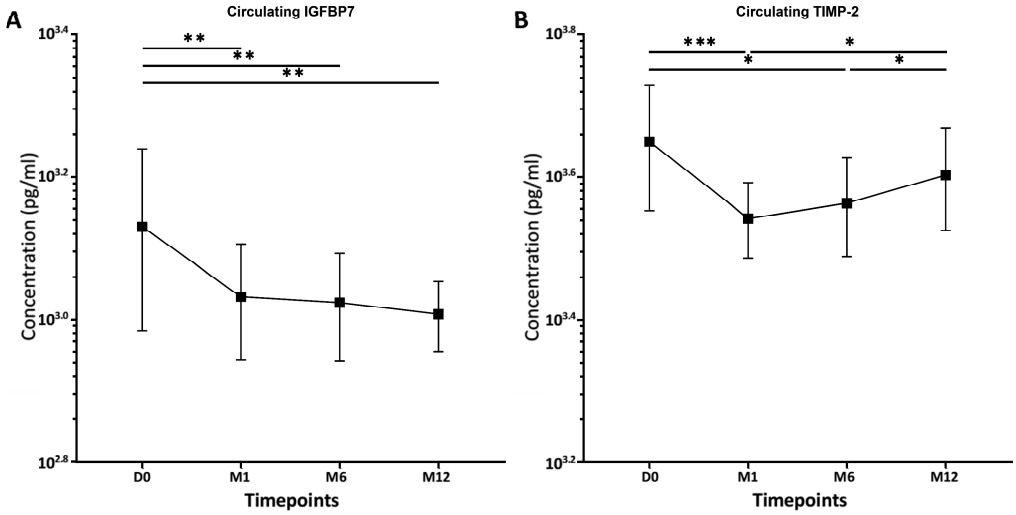

Figure 2. Circulating IGFBP7 (A) and TIMP-2 (B) have a different course after simultaneous pancreas-kidney transplantation. *IGFBP7 and TIMP-2 both decline shortly after simultaneous pancreas-kidney transplantation. Subsequently, IGFBP7 remains lower while TIMP-2 increased one year after transplantation. Levels are depicted as logarithmic values (mean ± SD). D0 = before transplantation, M1 = 1 month after transplantation, M6 = 6 months after transplantation, M12 = 12 months after transplantation. * p < 0.05, ** p < 0.01, *** p < 0.001.*

Discussion

This study shows that circulating levels of IGFBP7 and TIMP-2 are significantly higher in type-1 DM patients, compared with healthy controls. In type-1 DM patients with impaired kidney function (DM≤30), circulating levels of IGFBP7 further increased, most likely due to the fact that higher IGFBP7 is predominantly associated with impaired kidney function. This is supported by lower levels of IGFBP7 in SPKT recipients and the rapid persistent decline of IGFBP7 in the first year after a successful SPKT. TIMP-2, interestingly, does not significantly differ between DM≤30 patients and SPKT recipients. This is in accordance with the gradual return to pretransplant levels of TIMP-2 after SPKT. Therefore, TIMP-2 is suggested to be associated with chronic vascular injury, next to glomerular filtration.

Interestingly, circulating TIMP-2 remained high in type-1 DM patients who received a SPKT, although TIMP-2 did have a weak correlation with eGFR. High circulating TIMP-2 levels in DM may therefore be the result of diabetes related systemic factors, next to altered glomerular filtration. As shown in *Supplementary Figure 1*, the fractional excretion of TIMP-2 was comparable between transplanted DM patients (KTA and SPKT) and HC and we did not find a correlation between creatinine corrected urinary levels and circulating levels of IGFBP7 and TIMP-2. This suggests that the higher levels of TIMP-2 in transplanted patients are not due to altered excretion, but may be the consequence of increased systemic

production and/or altered systemic dynamics and metabolism. The longitudinal study supports this hypothesis and shows similar circulating TIMP-2 levels one year after transplantation, compared with end-stage renal disease. Previously, TIMP-2 is described to be higher in chronic vascular injury. Higher levels of TIMP-2 have also been shown to correlate with interstitial fibrosis in patients with chronic allograft damage.[19] In contrast with circulating levels, urinary levels did not demonstrate differences between the groups in the cohort. Next to systemic production of IGFBP7 and TIMP-2, urinary levels are also highly dependent upon differences in renal excretion. A recent study showed urinary IGFBP7 and TIMP-2 to increase, due to increased filtration, decreased tubular reabsorption and leakage of IGFBP7 and TIMP-2 in the proximal tubule.[20] In addition, urinary levels may change due to higher suggested TIMP-2 levels locally in the kidney.[21]

Next to altered levels of TIMP-2 due to diabetes and kidney function, TIMP-2 may be higher in transplant recipients in general. Previous research showed kidney transplant recipients to have higher TIMP-2 levels in plasma, compared with healthy controls.[22] This is in contrast with our data, since we observed comparable levels of IGFBP7 and TIMP-2 between DM>30 patients and type-1 DM patients, who received a kidney transplantation alone. Different immunosuppressive regimes within the SPKT and KTA group did not show a correlation with IGFBP7 and TIMP-2 levels. Additionally, Bicknell showed no difference in TIMP-2 levels between patients that received tacrolimus or ciclosporin.[23]

The specific role of IGFBP7 and TIMP-2 in the etiology of the different types of kidney damage remains a subject of discussion. Both IGFBP7 and TIMP-2 are often described as indicators of kidney damage. However, it is increasingly recognized that IGFBP7 and TIMP-2 also play an active role in the etiology of kidney damage. TIMP-2 is considered to play a role in both the occurrence and the progression of renal lesions in DM patients.[24] IGFBP7 may serve as a factor in TGF-β1-induced tubular injury in DN.[25] In addition, IGFBP7 is suggested to play a role in hyperglycemia-related podocyte proliferation, by the TGF-β1/Smad pathway.[26] This is an interesting finding, since we found levels of circulating IGFBP7 and TIMP-2 to be elevated in DM with a preserved kidney function. Therefore IGFBP7 and TIMP-2 may be particularly interesting biomarkers in the context of this early stage of damage in DM patients. It would be interesting to investigate IGFBP7 and TIMP-2 levels in an even earlier stage of DM with micro-albuminuria to assess the added value of IGFBP7 and TIMP-2 in the early diagnosis of DM and prognosis of kidney function in these patients.

The main strength of this study is the assessment of both circulating and urinary levels in a cross-sectional and longitudinal cohort. This approach facilitates a better differentiation between kidney function related changes and the association with other factors, such as chronic vascular injury. Additionally, the KTA group acts as a control group for the SPKT group and differentiates between the effect of improved kidney function and improved glucose regulation.

This study has several limitations. Although the cohort is a well-defined cohort, group size was limited. In addition, the influence of impaired kidney function in transplant recipients could not be observed. Therefore, a group of KTA or SPKT patients with deteriorated kidney function would be interesting to study as well.

Conclusions

Circulating IGFBP7 and TIMP-2 are significantly higher in patients with DM. IGFBP7 shows to be largely dependent upon glomerular filtration and consequently return to normal levels, after a successful kidney transplantation. In contrast, circulating TIMP-2 levels remain higher, despite kidney transplantation and restoration of endogenous insulin secretion, suggesting a role in ongoing chronic and/or persistent vascular injury.

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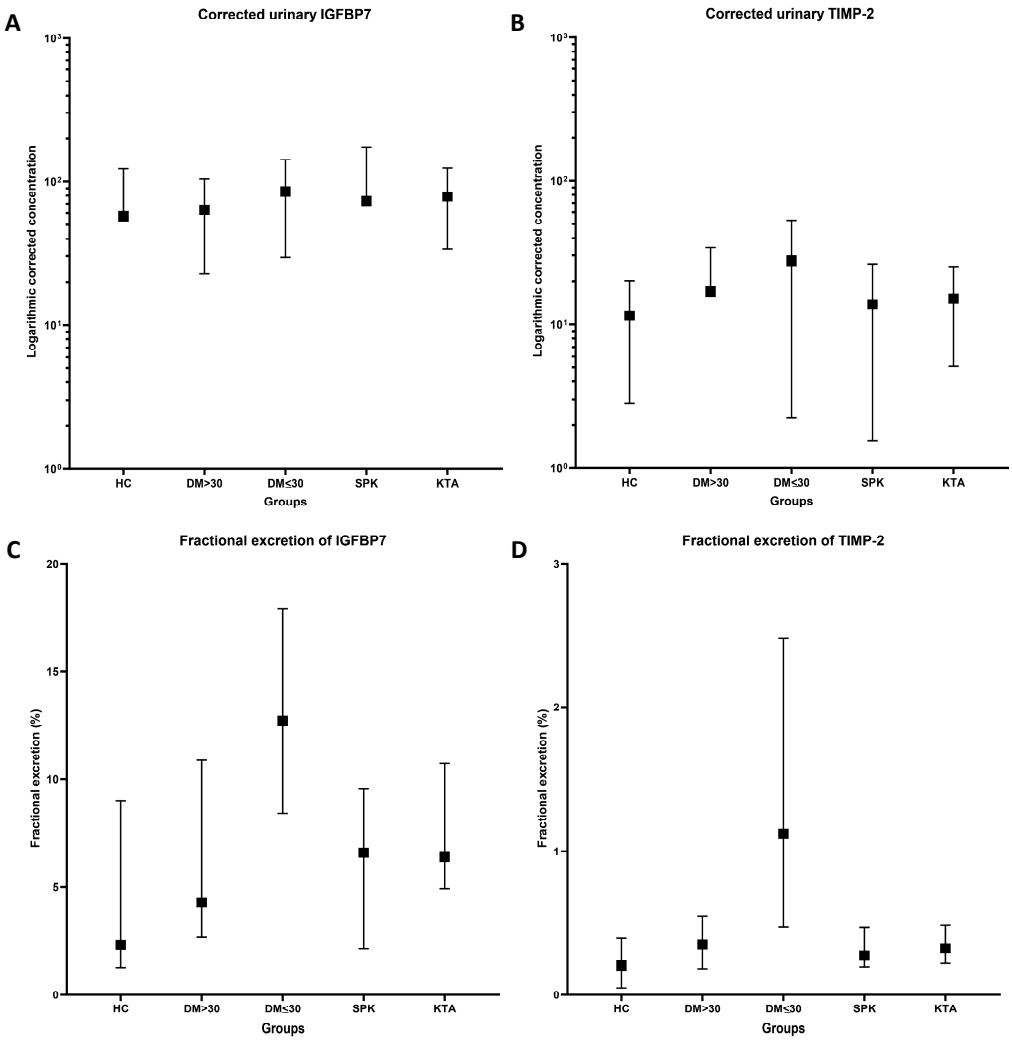
Supplementary information



Supplementary Figure S1. Urinary levels and fractional excretion of IGFBP7 (A and C) and TIMP-2 (B and D). *Urinary levels of IGFBP7 and TIMP-2 are comparable between the groups in the cohort. Fractional excretion of TIMP-2 was comparable between healthy controls (HC), type-1 DM patients with an eGFR >30ml/min/1.73m^2 (DM>30), and type-1 diabetes patients who received a simultaneous pancreas-kidney transplantation (SPKT) or a kidney transplantation alone (KTA).*