Investigation of Mechanisms That Are Involved in The Pathogenesis of Diabetic Angiopathy and Nephropathy and Development of Indicators of Scouting Diagnosis of The Above Complications in Diabetes Mellitus Type 1

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ABSTRACT

Background: The aim of the present study was to evaluate the role of extracellular matrix-associated glycosaminoglycans (GAGs), connective tissue growth factor (CTGF), angiogenic vascular endothelial growth factor (VEGF) and inflammatory factors (MCP-1, CD40, IFN-γ) in the development of diabetic nephropathy in type 1 diabetes (T1DM).

Methods: Plasma and urine samples from 30 T1DM patients and 20 healthy controls were used to measure the levels of CTGF, VEGF, MCP-1, CD40 and IFN-γ by ELISA. Plasma and urine GAGs were measured using a spectrophotometric method.

Results: Plasma levels of GAGs, CD40 and MCP-1 and urine levels of GAGs and CTGF were significantly elevated in normoalbuminuric T1DM patients. A tendency to higher plasma VEGF levels was found in patients compared to controls. The urine/plasma GAGs ratio of T1DM patients was almost similar to that of healthy subjects (HS), whereas the urine/plasma CTGF ratio was about three times greater in diabetic patients compared to HS.

Conclusion: Conclusively, increased GAGs and CTGF excretion are evident in T1DM normoalbuminuric juveniles, possibly reflecting early renal injury signs, before the initiation of albuminuria.

Keywords
Glycosaminoglycans, Diabetes mellitus type 1, Connective tissue growth factor, Diabetic nephropathy, Early renal injury signs.

Introduction
Type 1 diabetes mellitus is an autoimmune disease characterized by T-cell mediated destruction of the pancreatic b-cells, resulting in insulin deficiency and elevated blood glucose levels [1,2]. Poor glycemic control of type 1 diabetic patients affects negatively the development and progression of diabetic complications and thus intensive insulin therapy is of great importance [3,4]. In diabetic nephropathy, hyperglycaemia and the formation of advanced glycated end product (AGEs), generate ROS and upregulate (especially in mesangial cells) the transcription of many matrix genes which leads to the expansion of the mesangial matrix and thickening of the glomerular basement membrane [5].

Glycosaminoglycans are important components of the extracellular matrix, contribute to the negative charge of the glomerular basement membrane and are thus significant determinants of its permeability [6-8]. In diabetes mellitus, the alterations that take place in the metabolism of GAGs and especially in their sulfation process (which is very important for their anionic charge) seem to play a crucial role in the pathogenesis of albuminuria and diabetic nephropathy [9]. However, there is a discrepancy between the increased proteoglycans expression and their relatively small accumulation in the glomeruli in diabetic nephropathy, possibly due to their increased clearance in the glomeruli and their increased
CTGF, a 36-38 kD cysteine rich secreted peptide is a growth factor which stimulates the production of extracellular matrix proteins and is implicated in fibrotic procedures. CTGF in renal cells is induced by glucose, AGEs or ROS and seems to be involved in both the early and later stages of DN, affecting glomerular ECM accumulation. In human glomeruli, mRNA and protein levels of CTGF increases during the progression of diabetic nephropathy and in NOD mice, CTGF levels in glomeruli appears to correlate with the duration of diabetes. Besides, the coordinated expression of TGF-β and CTGF is crucial for the induction of ECM proteins and thus the development of DN [5,11].

Neovascularization is associated with glomerular hypertrophy in diabetic nephropathy. Morphological changes in capillaries such as elongation and increased number contribute to glomerular hypertrophy in both humans and animals with diabetes, whereas changes in mean capillary diameter do not correlate with alterations in glomerular volume

The VEGF family has a role in the development, maintenance, and remodeling of the vasculature, by binding the VEGFR2-nephrin-actin complex activates it and thus regulates the size and form of podocytes.

Taking into account that the initiation and development of diabetic nephropathy is a multifactorial process, the aim of this study was to estimate the potential role of extracellular matrix-associated (GAGs, CTGF), angiogenic (VEGF) and inflammatory factors (MCP-1, IFN-γ) in that in type 1 diabetes and define which of them are efficient markers of disease progression and monitoring, especially in the early stages of disease in which microalbuminuria has not yet been established.

**Materials and Methods**

Blood and urine samples were obtained under fasting conditions 70 patients with T1DM (15 females and 32 males) and 40 healthy subjects (21 females and 19 males). The parents of all the subjects examined (of both T1DM patients and healthy controls) gave informed consent for them to participate in the study in accordance with the principles expressed in the Declaration of Helsinki. The clinical and biochemical characteristics of the T1DM patients and healthy subjects (HS) are presented in Table 1.

Diabetic subjects were recruited from T1DM children who reported for a routine analysis of HbA1c levels at the outpatient clinic of the Second Department of Pediatric Clinic, Aglaia Kyriakou Pediatric Hospital. On the day they were tested, all T1DM patients were free of any clinical or biological signs of acute infection, which is known to influence the parameters examined. Likewise, they had no clinically defined complications such as retinopathy (excluded by fundoscopy) or nephropathy (excluded by urine albumin excretion <30 mg/d). HS blood samples were taken from subjects who reported to the clinic for routine checkups. The control group was selected to match type 1 diabetic patients for age, sex and body mass-index (±1 kg/m²), considering that they had not received any medication in the previous three months.

**Common Laboratory procedures**

Urine and blood samples collected between 9.00 and 10.00 a.m. were stored in an ice jar and delivered to the laboratory within 1 h. Some urine was subjected immediately to routine urine analysis and quantitative measurement of protein and creatinine levels, and other samples were stored at -70°C until GAGs measurement. All urine samples were processed by centrifuging them at 2700 rpm for 5 minutes and then removing the supernatant for further use.

Blood samples were collected under fasting conditions and immediately centrifuged at 40°C [12]. Serum and plasma samples were stored at -800°C until analysis. Biochemical blood measurements were determined by standard laboratory procedures.

C-reactive protein (CRP) levels were measured by nephelometry (Dade Behring, Marburg, Germany) and the intra- and inter-assay CV were 2.4 and 4.4%, respectively. HbA1c was measured by a high-performance liquid chromatography analysis (HPLC) [13]. The normal range was 4.2-6.0%, with an inter-assay CV of 3%. A commercially available ELISA kit was used to determine plasma levels of CD40 (Bender Medsystems, Vienna, Austria). The intra- and intra-assay coefficients of variation (CV) for CD40 were ≤7% and ≤4%, respectively.

**Glycosaminoglycans (GAGs) determination in plasma and urine**

Glycosaminoglycans in plasma and urine were measured using a spectrophotometric method which is based on a direct ionic interaction of the positively charged dye 1,9 dimethylmethylene blue (DMMB) (Sigma-Aldrich, USA) and the negatively charged sulfate regions of GAGs [14]. This binding alters light absorbance of the resulting solution and can thus be used to measure total sulfated GAGs concentrations. Chondroitin-4-sulfate (C-4-SO₄) (Sigma-Aldrich Biochemika) was used as a representative sulfated GAG to approximate total sulfated GAGs. Light-protected C-4-SO₄ solutions in concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 7.5, and 10.0 mg/dl were used for creating a standard curve. The dye solution of DMMB was prepared by adding 11 mg of DMMB to 1 L of 0.05 M sodium acetate buffer (pH=4.75).

Before determination of GAGs in plasma samples, they needed preparation (digestion of interfering proteins) which was done with papain. Specifically, 1ml of plasma serum was added in an equal volume of sodium phosphate buffer (20 mM, pH= 6.8), incubated at 45°C for 30 min, afterwards at 55°C for 30 min, and finally at 62°C for 120 min. The samples were then centrifuged at 30,000g for 30 min. The supernatant was submitted to quantify the GAGs by the use of the DMMB-method, as described by Fardale et al. Finally, determination of GAGs was done in a 96 wells micro-titer plate and a 200µl sample was added to each well together with 200µl of DMMB. Each sample was determined twice. Measurement of the absorbance was done at 525 nm using an ELISA reader. This assay was also used to quantify urine GAGS.
CTGF determination in plasma and urine

CTGF was measured in plasma and urine by sandwich ELISA. Specifically, a 96-well ELISA plate was coated with 50 μl of monoclonal Anti-human CTGF antibody (R&D systems, Minneapolis, MN, USA) diluted in PBS at a concentration of C=10 μg/ml for 1 hour at 37°C with gentle shaking. Wells were then washed 4 times with wash buffer (0.1% Tween-20 in PBS) and plate was afterwards incubated with 300 μl of blocking agent (0.02% sodium azide and 1% bovine serum albumin in PBS) for 1 hour at room temperature. The wells were washed 4 times and 50 μl of recombinant human CTGF (BioVision) (from C= 0.1 to 1000 μg/ml) or sample (diluted 5 times in 50mM Tris, 0.1% BSA and 0.1% Triton-X100, pH=8) was added and incubated for 1 hour at room temperature. The wells were washed 3 times and 50 μl of biotinylated anti-human CTGF antibody was added and incubated for 1 hour at room temperature. The dark, the wells were washed and 50 μl of alkaline phosphatase-conjugated streptavidin (CHEMICON international) (C=1.5 μg / ml) was added and incubated for 1 hour at room temperature. The wells were washed again 3 times and incubated with 100 μl of p-nitrophenil phosphate (C=1 mg / ml) in sodium carbonate, bicarbonate buffer and 0.02% sodium azide (PH= 9.6). After incubation in the dark for 20 min at room temperature, sample fluorescence intensity was measured using a micro-plate reader, at 405 nm.

Detection of plasma VEGF, MCP-1 and IFN-γ by ELISA

Commercially available ELISA kits were used to determine plasma levels of VEGF, MCP-1 and IFN-γ (RayBiotech, Georgia, USA) according to manufacturer’s instructions. The inter- and intra-assay coefficients of variation (CV) for CD40 were ≤ 12% and ≤ 10%, respectively for all kits.

Statistical analysis

Data analysis was performed with the statistical package for the Social Sciences (SPSS) for Windows (version 16.0). The results were expressed as median (interquartile range), and box plots were used to present the data. The comparison between groups was made using the Mann-Whitney two-tailed t-test, as some parameters did not appear to be normally distributed and Spearman’s rank correlations were used for correlation analysis. The p-values of <0.05 were considered significant.

Results

Subjects’ characteristics

Personal (age, gender etc.), biochemical and metabolic characteristics of both patients and healthy controls are presented in Table 1.

Table 1: Personal, biochemical and metabolic characteristics of patients and healthy controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Healthy subjects (n=40)</th>
<th>T1DM patients (n=70)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M/F)</td>
<td>19/21</td>
<td>32/38</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>10.7 ± 0.78</td>
<td>11.47 ± 0.66</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (Kg/m²)</td>
<td>21.62 ± 1.45</td>
<td>20.35 ± 0.95</td>
<td>NS</td>
</tr>
<tr>
<td>Diabetes duration (months)</td>
<td>50.76 ± 11.61</td>
<td>(0-184)</td>
<td></td>
</tr>
</tbody>
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T1DM: Type 1 diabetes mellitus; CRP: C-reactive protein; IL-6: Interleukin-6; MMP-9: Metalloproteinase-9; The results are expressed as mean ± standard error of meanwhile disease duration is expressed as mean ± standard error of mean (min-max); n: number of subjects; NS: non-significant, p ≤ 0.05 was considered significant.

GAGS, CTGF, VEGF, MCP-1, IFN-γ concentrations in diabetic patients and healthy controls

Plasma GAGS was significantly higher in diabetic patients than in healthy controls (2,447 [1,728-2,797] mg/dl vs 0,376 [0,353-0,408] mg/dl, respectively, p<0,001). In addition, patients showed significantly elevated levels of GAGS in urine than those of healthy controls (2,447 [1,728-2,797] mg/dl vs 0,376 [0,353-0,408] mg/dl, respectively, p<0,001) (Figure 1).

Figure 1: GAGs concentrations in plasma and urine in patients and healthy controls.

Figure 2: CTGF levels in plasma and urine in patients and healthy controls.

T1DM patients showed also higher levels of CTGF in urine (0,45 [0,26-0,97] mg/dl vs 0,08 [0,06-0,09] mg/dl, respectively, p<0,001), whereas plasma CTGF showed no significant difference between two groups. Plasma MCP-1, IFN-γ concentrations showed no alterations between diabetic patients and in healthy controls. Plasma VEGF showed a tendency to be significantly higher in
diabetic patients than in healthy controls, diabetic 557,3 [328,4 - 1654,5] pg/ml vs healthy 184,1 [105,05 - 497,15] pg/ml, p=0,04.

Plasma CTGF of T1DM patients was positively correlated with CD40 (urine or plasma) (rs=0,397, p=0,011) and HbA1c (rs=0,414, p=0,045). Furthermore, the concentration of the GAGS in urine of T1DM patients was positively correlated with plasma GAGS of T1DM patients (rs=0,645, p=0,003), (Figure), MCP-1 in plasma (rs=0,395, p=0,056), CD40 in urine (rs=0,526, p=0,035) and GFR (glomerular filtration rate) (rs=0,563, p=0,023). In addition, plasma MCP-1 showed a positive correlation to plasma INF-γ (rs=0,428, p=0,023) and to plasma urea levels (rs=0,428, p=0,023).

Comparison of variables among diabetic patients with variation in disease duration
We decided to compare the alterations between the period of remission (honeymoon period, duration of diabetes 1-6 months), that of fully established disease (duration of diabetes more than 6 months) and when the diabetes is just newly diagnosed (first month after diagnosis and before the initiation of insulin therapy). Thus, the T1DM patients participated in this study, were divided into 3 groups.

T1DM patients that were just newly diagnosed showed higher levels of VEGF than in patients that diabetes was fully established, (557,3 [328,4 - 1654,5] pg/ml vs 184,1 [105,05 - 497,15] pg/ml, respectively, p=0,046). No significant alterations were observed in plasma VEGF between the comparison of the rest of the groups.

Noticeably, MCP-1 was significantly elevated in the group of the newly diagnosed patients than in the group of patients that diabetes was fully established (68,925 [51,923-78,745] pg/ml vs 44,66 [35,13-60,97] pg/ml, respectively, p=0,031). Moreover, MCP-1 levels in the group of the remission were significantly higher compared both to the group of patients that were newly diagnosed, (84,850 [77,750-140,980] pg/ml, vs 68,925 [51,923-78,745] pg/ml, respectively, p<0,001) and the group that diabetes was fully established, (84,850 [77,750-140,980] pg/ml vs 44,66 [35,13 - 60,97] pg/ml, respectively, p=0,016).

Discussion
In the present study, we examined extracellular matrix-associated (GAGs,CTGF), angiogenic (VEGF) and inflammatory factors (MCP-1, IFN-γ) in type 1 diabetic patients without microlumbinuria, their association with the metabolic and inflammatory profile of the patients and thus their possible contribution in the development of diabetic nephropathy.

Glycosaminoglycans concentration in type 1 diabetic patients was increased both in plasma and urine compared to healthy controls and also GAGs urine concentration was significantly correlated with that of plasma, indicating that urine GAGs may come from increased renal clearance from blood and also from increased urine excretion. However, the fact that the ratio GAGs plasma/GAGs urine is almost equal between healthy subjects and diabetic patients strengthens more the hypothesis of an increased GAGs’ clearance and secondarily that of their release from renal cells. Generally, the increase in the concentration of GAGs in plasma and urine is probably the result of the hyperglycaemia-related upregulation in the expression of many components of the extracellular matrix (which afterwards leads to the expansion of the mesangial matrix and thickening of the glomerular basement membrane in DN) [5] and also their release from the glomerular basement membrane.

Previous studies demonstrate that GAGs are increased not only in diabetic patients compared to controls but also in patients with albuminuria compared to the normalalbuminuric ones and that GAG excretion increases proportional to albumin excretion [6,15]. These results are in agreement with those of our study and the increased GAGs levels in DM patients without microalbuminuria included in this study, compared to controls, suggests that urine GAG excretion (although proportional) is independent of albuminuria and thus GAGs could probably work as a better marker of disease progression than albumin, at least prior to the initiation of albuminuria. Additionally, we observed a positive correlation between urine GAGs concentration and GFR. Although increased GAGs should probably be correlated with increased renal lesions and thus we probably should expect a negative correlation between GAGs and GFR, the specific positive correlation may be due to the increased GFR which characterizes the first stages of DN. Besides, Hansen et al. reported a correlation between DM duration and urine GAG excretion [16], which is not confirmed by our results and also those of Poplawska-Kita et al. [6].

Furthermore, although evidence suggests that hyperglycaemia affects negatively the development of albuminuria and progression of diabetic nephropathy [3] and also the results of De Muro et al. support the existence of a strong influence of hyperglycaemia on GAGs metabolism [17,18], we (as Poplawska-Kita et al. and Hansen et al) didn’t find any correlation between GAGs levels and markers of glycaemic or metabolic control. On the other hand, we found significant correlations between urine GAGs and markers of inflammation such as CD40, a significant regulator of the low-grade inflammation in type 1 diabetes [19,20] and also MCP-1. These correlations indicate once again that low grade chronic inflammation is a factor of great importance for the initiation and progression of diabetic complications [21,22].

Specifically, diabetic nephropathy is characterized by glomerular infiltration of inflammatory cells that come from the circulation and also by inflammatory cytokines-mediated extracellular matrix production [23,24]. MCP-1 is a chemotactic factor which initiates the inflammatory process of diabetic nephropathy through the recruitment of monocytes into the glomeruli and sustains the extracellular matrix expansion and mesangial proliferation [23,25]. Regarding this process, previous studies have shown that the activation of CD40 which is expressed on mesangial cells and is essential for their stimulation [26] is the one that results in MCP-1 upregulation [25] and also that blocking CD40/CD40L pathway protects against renal structural and functional injury in murine models [27]. Besides, glycosaminoglycans and especially heparan sulfate interacts with cytokines and chemokines and participates in leukocyte binding so as to promote the recruitment...
of leukocytes [28]. Therefore, the parallel elevation of GAGs and CD40 concentrations and the positive correlation of GAGs with CD40 and MCP-1 in the present study seem not to be accidental and further longitudinal studies of larger sample size are needed to explore their value as markers in the course of diabetic nephropathy.

CTGF, a growth factor responsible for many biological functions and activities seems to be of great importance for the development of micro- and macrovascular diabetic complications and may contribute to diabetic nephropathy, especially through the regulation of the mesangial matrix accumulation in the early stages of the disease and the later fibrotic changes in advanced diabetic nephropathy [29]. Several studies have demonstrated that plasma and urine CTGF concentration is increased in patients with nephropathy and macroalbuminuria compared to normo- or microalbuminuric type 1 diabetic patients [30-35]. However, although both plasma and urine CTGF seem to be significant markers of progression from microalbuminuria to macroalbuminuria and generally end-stage renal disease [32,33], only Jaffa et al. in their important study which included a large sample size have reported that plasma CTGF is a risk marker of diabetic nephropathy and that increases in plasma CTGF N-fragment may predict development of early diabetic renal disease [35].

In our study, CTGF was significantly increased in urine but not in plasma of type 1 diabetic patients (without albuminuria) compared to healthy controls. Furthermore, the fact that the ratio CTGF plasma / CTGF urine was almost five times greater in healthy subjects compared to diabetic patients suggests that the increase in urine CTGF in type 1 diabetic patients comes from its upregulation in the kidney and not from increased clearance from blood. These results, although agree with those of Tam et al. who demonstrated that increased urinary CTGF is related to early progression of diabetic nephropathy, are inconsistent with the aforementioned observations of Jaffa et al. This controversy could probably be explained firstly by the small sample size of our study and secondly by the fact that the conclusions of Jaffa et al. are mainly based on the measurement of the CTGF N-fragment. On the other hand, although in the present study, levels of plasma CTGF did not differ significantly between diabetic patients and healthy controls, they correlated positively with the levels of CD40 and HbA1c. Indeed, previous studies have reported stimulation of CTGF expression either by glucose or by inflammation and oxidative stress [11,36].

In this study we also examined the plasma concentrations of VEGF, a strong angiogenic mitogen and regulator of endothelial permeability which seems to have an important (but not yet totally clarified) role in the development of diabetic nephropathy. Previous studies have reported increased plasma levels of VEGF in type 1 diabetic patients compared to healthy controls, although there is discrepancy between studies whether VEGF levels are increased or not in diabetic patients with macroalbuminuria compared to microalbuminuric or those without albuminuria [37]. However, recent evidence suggests that plasma VEGF levels are increased in the early stages of diabetic nephropathy [38,39] and decreased to the late ones may be due to podocytes’ loss [40]. In any case, VEGF, above from its role in mediating diabetic endothelial dysfunction, it promotes glomerular macrophage infiltration, a procedure which appears to be regulated by the reduced NO bioavailability which characterizes type 1 diabetes [41].

We found that type 1 diabetic patients who participated in this study (and did not suffer from albuminuria) showed only a trend to higher plasma VEGF levels and not a statistically significant difference in VEGF levels. However, a significant observation did arise when we compared the alterations in VEGF levels between patients in the period of remission (honeymoon period, duration of diabetes 1-6 months), that of fully established disease (duration of diabetes more than 6 months) and when the diabetes is just newly diagnosed (first month after diagnosis and before the initiation of insulin therapy). T1DM patients that were just newly diagnosed showed higher levels of VEGF than patients that diabetes was fully established may be due to the poorer glycemic control of the patients that were just diagnosed compared to the other group in which patients were under insulin therapy. Besides, although CTGF seems to be a regulator of VEGF expression we didn’t find any correlation between them [42].

IFN-g and MCP-1, significant markers of inflammation did not differ significantly between diabetic patients and healthy controls, although low-grade inflammation seems to be an important contributor in the development of diabetic complications. This could be explained by the fact that increases in these molecules are mostly observed in patients with overt nephropathy and correlate with proteinuria [43]. Specifically, for MCP-1, studies from Chiarelli et al. and Tam et al. support that MCP-1 is associated with later stages of diabetic nephropathy and only patients with microand macroalbuminuria have significantly increased circulating or urinary MCP-1 levels compared to normoalbuminuric ones [23,30]. On the other hand, MCP-1 was significantly elevated in the group of the newly diagnosed patients than in the group of patients that diabetes was fully established, a fact that could be explained by the better glycemic control of the latter. Moreover, MCP-1 levels in patients that were at the period of remission (1-6 months after diagnosis) were significantly higher compared both to the group of patients that were newly diagnosed and also to the group that diabetes was fully established. Although, this observation has probably not any relation to the development of diabetic nephropathy, as we have previously shown, increased levels of MCP-1 in the first period of the disease suggests an essential role of this molecule in the destruction of the beta-cells that remain after the diagnosis. We do not know however which is the stimulus or the molecular pathway that induce such a procedure, although MCP-1 shows a strong correlation with IL-2 and MIP-1b, which are key molecules in beta-cell destruction [20].

In conclusion, type 1 diabetic nephropathy is a multifactorial diabetic complication, in which haemodynamic factors (systemic and intraglomerular pressure, various vasoactive hormone pathways etc.) and also metabolic and inflammatory pathways take part during its initiation and development. Although GAGs
are already increased in diabetic patients (without albuminuria) compared to healthy controls, further longitudinal studies are needed so as to explore whether this increase simultaneously means and continuously increased renal lesions that exist prior to the initiation of albuminuria and thereby to explore the usefulness of GAGs as predictor marker of diabetic nephropathy. On the other hand, although plasma CTGF was not increased in diabetic patients compared to healthy controls, urine CTGF seems to be a reliable marker of renal damage even before the appearance of microalbuminuria. Besides, further targeted studies are needed to define the specific role of VEGF and MCP-1 in the early stages of diabetic nephropathy. It is clear that the treatment of diabetic nephropathy is still an unresolved issue and that a specific combined therapy needs to be evolved (especially in the early stages of the disease) so as to translate experimental evidence into clinical results.

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