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*Differential expression of Tissue Factor (TF) in
calcineurin inhibitor-induced nephrotoxicity and rejection*

- Implications for development of a possible diagnostic marker

C. Österholm¹, B. Veress², M. Simanaitis²,

U. Hedner³ and H. Ekberg¹

¹Department of Nephrology and Transplantation, ²Department of Pathology, Lund
University, Malmö University Hospital, Malmö, Sweden

³Tissue Factor/Factor VII Research, Novo Nordisk A/S, Måløv, Denmark

Address correspondence to:

Dr. Henrik Ekberg

Department of Nephrology and Transplantation,

Lund University, Malmö University Hospital,

S-205 02 Malmö, Sweden

Tel: +46 40 33 37 41

Fax: +46 40 33 73 35

E-mail: henrik.ekberg@kir.mas.lu.se

Abstract (word count 381)

Deposition of fibrin in the form of fibrinoid necrosis is a common feature of severe acute renal allograft rejection. The role of the coagulation system and its initiator tissue factor (TF) during this process is, however, still poorly understood. In this study, we analyzed the expression of TF in 88 renal transplants afflicted with different forms of rejection and calcineurin inhibitor-induced nephrotoxicity, to see whether there was differential expression of this protein. TF immunoreactivity was evaluated semiquantitatively in six different renal structures: the podocytes, Bowman epithelium, the endothelium of the glomeruli, the brush border of tubular cells, the thin ascending loop of Henle, and small arteries/arterioles. The TF expression of normal renal tissue (n = 6) was restricted to the glomerular podocytes and Bowman epithelium, and to some extent the ascending loop of Henle. Renal allografts undergoing acute rejection (AR) of grades I–III, (n = 13, n = 17 and n = 12, respectively) did not show any altered TF expression in the glomeruli or vascular endothelium. In the ascending loop of Henle, a reduced expression could be seen (ARI, p = 0.015; and ARII, p = 0.043). TF staining of the brush border of renal transplants undergoing acute cyclosporin A (CsA) nephrotoxicity (n = 18) was significantly higher than in normal kidneys (p = 0.0003), as well as in transplants undergoing various degrees of acute rejection (ARI, p = 0.027; ARII, p = 0.0012; and ARIII, p = 0.0001). Tubular brush border-expressed TF was also evident in 10 of 15 allografts suffering from chronic CsA nephrotoxicity, compared to 4 out of 13 cases with chronic allograft vasculopathy (CAV), but the increase was not statistically significant relative to normal kidneys. The majority of the grafts afflicted with either of the two chronic conditions displayed a TF-positive arterial endothelium (CAV, p = 0.0034; and chronic CsA nephrotoxicity, p = 0.0026) relative to controls. In conclusion, these results indicate that vascular TF expression is not altered during acute rejection, but may be of importance in chronic allograft nephropathy. Furthermore, TF immunoreactivity in the tubular brush border

may be specific to acute CsA nephrotoxicity and might be used as a biomarker for this condition. Further studies are required to evaluate the possible role of brush border-expressed TF in the pathogenesis of CsA nephrotoxicity.

Keywords: tissue factor, calcineurin inhibitor, nephrotoxicity, acute rejection, chronic rejection

1. Introduction

Calcineurin inhibitors effectively prevent acute rejection and increase short-term renal allograft survival. However, long-term graft survival is not improved to the same extent and chronic allograft nephropathy (CAN) has emerged as a leading cause of graft loss, in addition to death with functioning graft. Various factors, such as prolonged cold ischemia time and reperfusion injury, and also viral infection, are believed to contribute to the process of CAN [1,2]. However, a recent study by Nankivell *et al.* has shown explicitly that the vast majority of renal grafts afflicted with CAN display lesions that may be associated with drug-induced nephrotoxicity, as a result of treatment with cyclosporin A (CsA) and tacrolimus [3,4].

Acute calcineurin inhibitor-induced nephrotoxicity (hereafter referred to as CsA nephrotoxicity) manifests clinically as elevated serum creatinin levels and decreased glomerular filtration rate (GFR), and can be reversed with appropriate management, i.e. dose reduction, or withdrawal of CsA, or by switching to a non-nephrotoxic drug. Chronic CsA nephrotoxicity, on the other hand, is an irreversible condition. The diagnosis mainly relies on histological findings in biopsies, such as hyalinization of arterioli, striped interstitial fibrosis and tubular microcalcification [3-5]. Although these findings are considered to be characteristic histological markers, the discrimination between chronic allograft vasculopathy (CAV) and chronic CsA nephrotoxicity remains a challenge, as the two conditions show certain clinical and histological similarities.

Changes associated with acute rejection are classified into three different grades (Banff grades I–III) according to the severity of mononuclear infiltration of the interstitium and tubuli [5]. Chronic allograft vasculopathy (CAV), also referred to as true chronic rejection in the Banff

classification, is characterized by progressive intimal hyperplasia of the arteries/arterioli and interstitial fibrosis [5].

Tissue factor (TF), the physiological initiator of blood coagulation, is normally present in the peripheral structures of organs and this distribution provides a hemostatic barrier in traumatic injury [6]. It is abundantly expressed in the vascular adventitia of blood vessels and moderately expressed in the media. Endothelial cells facing the lumen are totally devoid of TF. In the kidney, TF expression is restricted to the glomerular podocytes, Bowman epithelium and, to some extent, the tubular loop of Henle. Apart from its role in initiation of hemostasis, TF has also been identified as a mediator of arteriosclerosis, tumor metastasis and angiogenesis [7-9]. Several studies have also described an effect of coagulation factors (including TF) that increases inflammation as well as fibrosis [10,11].

Deposited fibrin (in the form of fibrinoid necrosis) is the end-product of the blood coagulation cascade and is characteristic of severe acute rejection of renal allografts; yet, the role of the coagulation cascade during this process is poorly understood. In the healthy body, the natural anti-coagulants predominate and strictly control the activities of the pro-coagulants, thus maintaining a non-thrombogenic environment. However, the presence of inflammatory mediators such as the cytokines TNF- α , IL-1 β and IL-6, has a propensity to shift the hemostatic balance in favor of coagulation and thrombosis (reviewed in [12]).

2. Objective

The objectives of this study were as follows. Firstly, we wanted to determine whether the intensity and distribution pattern of TF expression in renal allografts could be correlated to the presence and the severity of acute rejection and acute CsA nephrotoxicity. Secondly, we

aimed to determine whether the distribution of TF was different in allografts showing histological signs of CAV to grafts afflicted with chronic CsA nephrotoxicity.

3. Materials and Methods

3.1 Tissues

Biopsy material and surgical specimens were obtained from the Department of Pathology at Malmo University Hospital. Twenty biopsies, previously acquired by ultrasound-guided needle sampling, were selected randomly from each of six groups of diagnoses from the clinical database in addition to a control group. The conditions had previously been clinically suspected and histologically confirmed according to the Banff criteria [5]. The six groups were: groups 1–3, acute rejection (AR I–III); group 4, CAV; group 5, acute CsA nephrotoxicity; and group 6, chronic CsA nephrotoxicity. The control group (group 7, n = 6) included surgical specimens of healthy tissue from kidneys removed due to cancer. In order to obtain rapid diagnosis, renal biopsies are routinely heated in a microwave oven before embedding and immunostaining. The effect of this procedure, concerning possible unspecific immunostaining in various tissue components, was studied in the control kidneys by the use of microwave pre-treated as well as untreated tissue samples. Ethical committee approval and informed consent from patients were obtained.

3.2 Immunohistochemistry

A polyclonal rabbit antibody against human soluble recombinant TF (Batch 110996) was prepared at Novo Nordisk A/S, Bagsvaerd, Denmark. A biotinylated secondary goat anti-rabbit IgG antibody was purchased from Vector Laboratories Inc., CA. The procedure was performed on 3- μ m paraffin sections using the DAKO TechMate™ 500/1000 automatic system together with the DAKO ChemMate™ Detection Kit according to the manufacturer's

staining protocol, including microwave antigen retrieval (DAKO, Denmark). Chromogenic diaminobenzidine was used as substrate in the peroxidase reaction. The sections were counterstained with Mayer's hematoxylin and mounted in DPX. As a negative control, the antibody was omitted and replaced by serum. To verify the specificity of the antibody, it was also pre-absorbed with excess recombinant TF and then used for staining sections from control renal tissue as well as biopsies, according to the procedure described above (data not shown).

3.3 Evaluation of TF staining

The distribution of TF was assessed in the glomeruli (podocytes, Bowman epithelium and endothelium), in the tubular brush border and ascending loop of Henle, and also in the endothelium (EC) and smooth muscle cells (SMC) of the arteries/arterioles. Two investigators, blinded as to the group numbers, analyzed all biopsies in random order (one section per biopsy). The intensity and distribution of the immunoreactivity for podocytes was given the following scores: 0 = none, 1 = patchy, 2 = weakly to moderately diffuse, and 3 = strongly diffuse. For brush border: 0 = none, 1 = patchy weak, 2 = strong in one group of tubuli, and 3 = strong in more than one group of tubuli. For all other structures: 0 = none, 1 = weak to moderate, and 2 = strong immunoreactivity.

3.4 Statistics

The Kruskal-Wallis non-parametric test (Statview 4.5) was used to detect differences between the groups. Differences between individual groups were identified by post hoc testing according to Siegel *et al.* [13]. P-values less than 0.05 were considered to indicate statistical significance.

4. Results

4.1 Tissues

Twenty biopsies from each group with acute rejection grades I–III, acute and chronic CsA nephrotoxicity, and also CAV had been randomly selected for analysis. A second round of evaluation confirmed the diagnoses of CAV and chronic CsA nephrotoxicity. Four biopsies showing characteristics of both conditions were assigned to the chronic CsA nephrotoxicity group. Sections with an insufficient amount of tissue, or the presence of a strong nonspecific background staining due to microwave treatment, were excluded. Thus, the actual numbers of biopsies analyzed in the groups varied between 12 and 18.

4.2 Tissue Factor expression in the normal kidney

In the control group (n = 6), three tissue components were positive for TF antigen: the podocytes and Bowman epithelium of the glomeruli, both showing moderate to strong immunoreactivity (Fig. 1a), and the ascending loop of Henle, which was weakly or moderately stained (Fig. 1b). All other structures, including the brush border of the tubular epithelial cells and the glomerular endothelium, showed no immunoreactivity. The TF expression pattern was identical among the different normal specimens, except for scoring of the Bowman epithelium where a slight variation between weak and moderate staining could be observed. The TF scores of each group were compared with the scores of the corresponding renal structure in the control group. Staining of sections from the various groups with antigen-absorbed antibody revealed that the microwave pretreatment used during the routine rapid histological procedure gave rise to an unspecific granular cytoplasmic staining of the tubular epithelium and the cellular nuclei. The various tissue components were completely negative otherwise.

4.3 TF expression during acute rejection I–III and acute CsA nephrotoxicity

The allografts afflicted with AR I–III or acute CsA nephrotoxicity demonstrated no significant alterations with regard to TF expression in any of the structures examined in the glomeruli (Figs. 1a and 2a). The brush border of the tubular epithelial cells stained positively in cases of acute rejection and CsA nephrotoxicity, with varying intensity (Figs. 1c and 2b). A significant increase in TF expression in the brush border of the acute CsA nephrotoxicity group could be observed relative to all other groups. It was statistically significant also when compared to AR group I ($p = 0.027$), which had the highest TF scores of the acute rejection groups. All biopsies in the acute CsA nephrotoxicity group were positive (56% were heavily stained), whereas 25–54% were positive in the AR groups (none were heavily stained). The ascending loop of Henle showed reduced immunoreactivity in AR groups I ($p = 0.015$) and II ($p = 0.043$) (Figs. 1d and 2b). Blood vessels from the groups with acute rejection or acute CsA nephrotoxicity were completely negative (Fig 2c), as were the glomerular endothelia.

4.4 TF expression in allografts afflicted with CAV and chronic CsA nephrotoxicity

In the groups diagnosed as CAV and chronic CsA nephrotoxicity, a weak endothelial staining within the glomeruli could be detected in about half of the biopsies, $p = \text{n.s.}$ (Fig. 3a). The podocytes and Bowman epithelium showed normal staining patterns. Regarding the epithelial brush border, the majority of biopsies in the chronic CsA nephrotoxicity group were positive (10 out of 15), whereas in the CAV group the majority of the biopsies were negative (9 out of 13) (Fig. 3b). There were, however no detectable statistically significant differences between the groups, including the control group (chronic CsA nephrotoxicity vs. control, $p = 0.59$). Three of the four biopsies displaying signs of both CAV and chronic CsA nephrotoxicity showed positive staining of the tubular brush border. In the cytoplasm of the tubular epithelium, a brown granular staining was observed but this could not be evaluated with

certainty or be regarded as pathological, due to the routine microwave treatment of the biopsies. The epithelial cells of the Henle loops showed a normal immunostaining pattern (Fig. 3b). The small arteries were TF positive in all biopsies, with the exception of one in each group (CAV, $p = 0.0034$; chronic CsA nephrotoxicity, $p = 0.0026$) (Fig. 3c).

5. Discussion

The main finding of this study was the distinct TF staining of the brush border of the tubular epithelial cells in both acute and chronic CsA nephrotoxicity, and to a certain extent also in acute rejection. Furthermore, we observed a diminished immunostaining of the epithelium of the loop of Henle in allografts undergoing AR of grades I and II. A mild immunoreactivity was observed in the vascular endothelium in the CAV and chronic CsA nephrotoxicity groups. There was no detectable change in the expression of TF in the other tissue components.

Acute nephrotoxicity due to treatment with calcineurin inhibitors manifests as elevated serum levels of creatinine and decreased GFR, and, if there is no intervention, may progress to a chronic state, which is virtually irreversible. The chronic form of the condition is difficult to distinguish clinically, and its diagnosis relies instead on histological findings, which are sometimes difficult to distinguish from other potentially chronic disease states of the graft. One specific finding of this study was the definite TF staining seen in the tubular brush border of biopsies from the acute nephrotoxicity group. This staining was significantly stronger than in the control group, and also relative to all the groups undergoing acute rejection. The underlying cause of this heavy staining is unknown, but as the tubular cells are exceptionally susceptible to hypoxia, one possible explanation is that TF may be induced in this structure as a result of ischemia/reperfusion injury due to renal vasoconstriction and oxidative stress,

which are both features of CsA-induced nephrotoxicity. It is well known that fibrogenesis occurs in the form of interstitial fibrosis in both CAV and chronic CsA nephrotoxicity. Angiotensin II (Ang II), which is a regulator of the renin-angiotensin system (RAS) and a potent vasoconstrictor, has been implicated as an inducer of TF in monocytes, smooth muscle and endothelial cells [14-16], as well as being an important factor for the progression of interstitial fibrosis induced by cyclosporine A (CsA) [17]. It is not yet clear whether Ang II induces TF expression in the tubular brush border directly or as an effect of renal vasoconstriction and/or oxidative stress, and whether the expression may contribute to the tubular fibrogenesis induced by Ang II. Endothelin-1 (ET-1) is another molecule that is suspected of mediating CsA nephrotoxicity by acting as a vasoconstrictor and promoting interstitial fibrosis [18]. A recent study has shown that the TF/FVIIa complex is capable of inducing ET-1 expression in bovine aortic endothelium [19].

Although efforts were made to verify the diagnoses of CAV and chronic CsA nephrotoxicity in this study, we cannot be completely certain that some of the grafts that were diagnosed with CAV only did not have a component of CsA nephrotoxicity. It has been demonstrated recently that most renal allografts afflicted with CAN actually have an associated chronic CsA-induced nephrotoxicity [3,4].

The frequently observed fibrin deposition during allograft rejection indicates that the local hemostatic balance is disturbed. Previous reports have supported this notion by describing reduced levels of naturally occurring anticoagulants such as antithrombin III and proteins involved in the protein C pathway, protein C (PC), protein S (PS) and thrombomodulin [20,21]. Inhibition of fibrinolysis by reduced levels of tissue plasminogen activator (tPA) as well as induction of plasminogen activator inhibitor-1 (PAI-1) have also been reported

[22,23]. The presence of the pro-coagulant TF during renal allograft rejection or other pathological conditions of the graft has not been studied to the same extent. The results of this study indicate that TF is not induced in the vascular structures of the graft itself during acute rejection, not even in the more severe forms of acute rejection, and that upregulation of TF in these structures thus plays no clear role during the process. Reduced expression of naturally occurring anticoagulants therefore remains as a potential factor in fibrin generation during acute renal allograft rejection.

In chronic allograft nephropathy, on the other hand, TF may contribute to the widely noted fibrin deposition by being expressed on the vascular endothelium. The vast majority of the grafts diagnosed with CAV or chronic CsA nephrotoxicity displayed TF staining of the endothelium of arteries and/or arterioles. Previous studies have also implicated TF as an inducer of vascular proliferative changes [24,25], regardless of its participation in the coagulation process. Its presence in the grafts afflicted with CAV and chronic CsA nephrotoxicity raises the question of whether TF may act as a modulator of the vessel wall during these conditions.

Apart from the cellular components of the graft itself, there are other potential sources of TF that could induce the formation of fibrin, such as monocytes and/or platelets, or microparticles carried by the bloodstream. Monocytes are almost exclusively found in grafts undergoing severe acute rejection, and not in grafts afflicted with CsA nephrotoxicity. Due to the microwave pretreatment of the tissues used in this study, the limited numbers of monocytes were difficult to study because of their low cytoplasmic content and unspecific nuclear staining. TF immunoreactivity was therefore not assessed in these cells.

The ascending loop of Henle showed a reduced TF expression in the groups undergoing acute rejection of grades I and II. Bukovsky *et al.* previously described a depletion of glomerular TF in acute allograft rejection and found an association of this occurrence with a poor graft outcome [26]. Depletion of tubular TF has, however, not been described previously and its clinical significance remains unknown.

The occurrence of TF immunoreactivity of the tubular epithelial brush border may be of importance, however. According to studies by Lwaleed *et al.*, a variety of conditions such as cancer and inflammatory disease give rise to elevated levels of urinary TF (uTF) [27-29]. In view of the fact that the renal tubuli have been suggested to be a potential source of uTF [30], it is conceivable that the TF positivity at the brush border observed in our study indicates the production of TF by tubular epithelial cells. Unfortunately, we could not regard the brown granular cytoplasmic staining of tubuli as being truly positive, due to the microwave treatment of the biopsies. Thus, this tissue component was not included in the analyses. However, as a more intense degree of staining was observed in the chronic conditions, one might suspect that this represents TF synthesis by the epithelial cells. Future studies on non-microwaved biopsies may provide answers to these questions. It would also be of interest to screen urine samples from patients with suspected CsA nephrotoxicity vs. patients with acute allograft rejection, to evaluate a possible difference with regard to urinary TF content.

The specific increase in brush border immunoreactivity observed in the grafts displaying acute CsA nephrotoxicity prompted us to investigate whether the same differences could be observed in allografts undergoing CAV vs. chronic CsA nephrotoxicity. A large proportion of the allografts afflicted with CsA nephrotoxicity did indeed show positive TF staining, whereas the majority of allografts undergoing CAV were negative. The difference was,

however, not statistically significant. A large and sufficiently well-powered study might help to confirm this difference. However, the predictive value of brush border TF positivity for chronic CsA nephrotoxicity is uncertain.

Until other safe and effective drugs become available to replace calcineurin inhibitors, a balance between the risk of nephrotoxicity and the risk of acute rejection will have to be maintained. Expanding the repertoire of markers specific for the different conditions would be helpful, to discriminate between them and subsequently take proper measures. We believe that brush border-expressed TF and also uTF deserve further evaluation as potential markers of acute and chronic CsA nephrotoxicity.

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Legends

Fig. 1

- a) Control kidney. The glomerular podocytes and the Bowman epithelium are immunopositive. Note the lack of positivity in the brush border of the tubular epithelium (arrows).

- b) Control kidney. Moderate immunoreactivity of the Henle epithelium (arrows). Note the lack of immunostaining in the brush border of the tubuli (T), whereas the cytoplasm of the epithelial cells shows mild non-specific granular staining.

- c) Acute CsA nephrotoxicity. Strong brown immunostaining of the brush border within the tubuli (arrows).

- d) Acute rejection. No immunoreactivity of the Henle epithelium (H), but weak positive reaction of the tubular brush border (arrow). There is non-specific staining of the cellular nuclei due to microwave pretreatment during the rapid diagnostic procedure. (Immunoperoxidase for TF; original magnifications x320).

Fig.2

- a) Glomerular TF expression in allografts undergoing acute rejection or acute CsA nephrotoxicity. No significant differences were detected in the podocytes, Bowman epithelium or glomerular endothelial cells (gec), compared with the control group.

- b) Tubular TF expression in allografts undergoing acute rejection or acute CsA nephrotoxicity. A reduction in TF staining of the ascending loop of Henle could be

seen in AR group I ($p = 0.043$) and AR group II ($p = 0.015$). The tubular brush border in biopsies from the acute CsA nephrotoxicity group had a distinct staining that was significantly stronger than in all the other groups.

- c) TF expression of blood vessels in renal allografts undergoing acute rejection or acute CsA-induced nephrotoxicity. The blood vessels of all groups were completely negative.

Fig. 3

- a) Glomerular TF expression in renal allografts afflicted with chronic allograft vasculopathy (CAV) and chronic CsA nephrotoxicity. The podocytes and Bowman epithelium showed normal levels and patterns of expression compared to the controls. The glomerular endothelial cells (gec) displayed a weak positive staining in about 50% of the biopsies in both groups.
- b) Tubular TF expression in the CAV and chronic CsA groups. The ascending loop of Henle showed a normal expression. TF was expressed in the brush border of 4 biopsies in the CAV group ($n = 13$) and 10 biopsies in the chronic CsA group ($n = 14$) (n.s.), whereas the normal controls were completely negative.
- c) Arterial TF expression in the CAV and chronic CsA groups. Weakly positive staining was observed in almost all biopsies in both groups. The control group was negative.

Fig 2

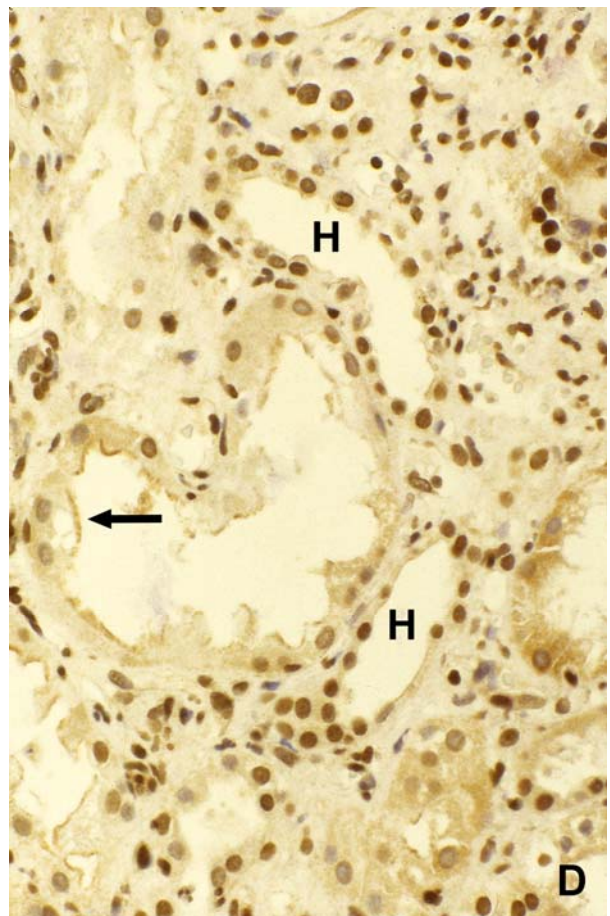
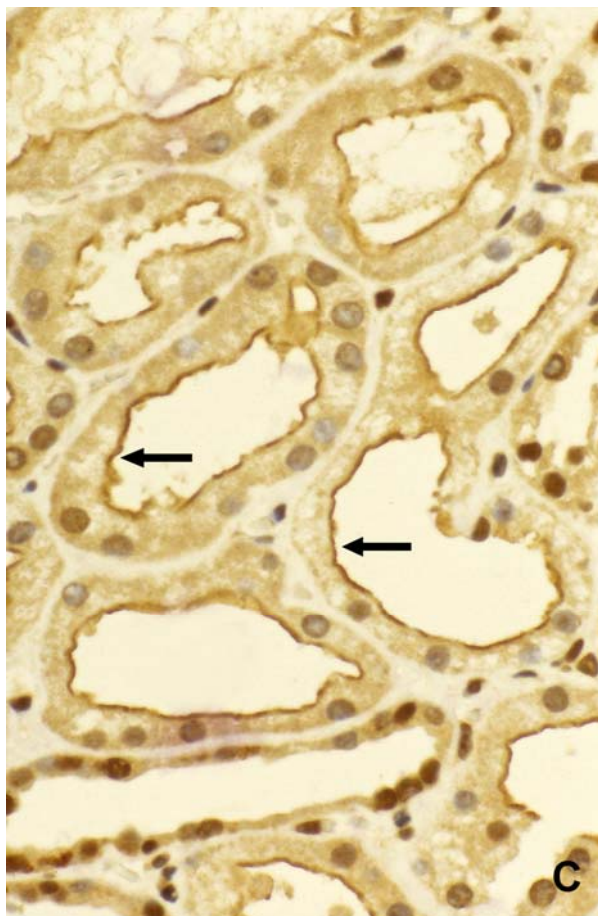
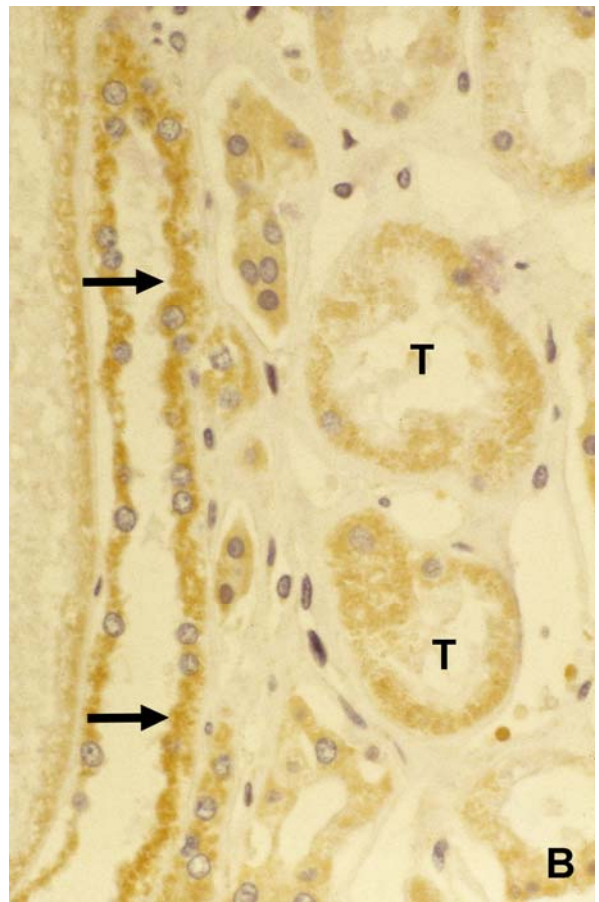
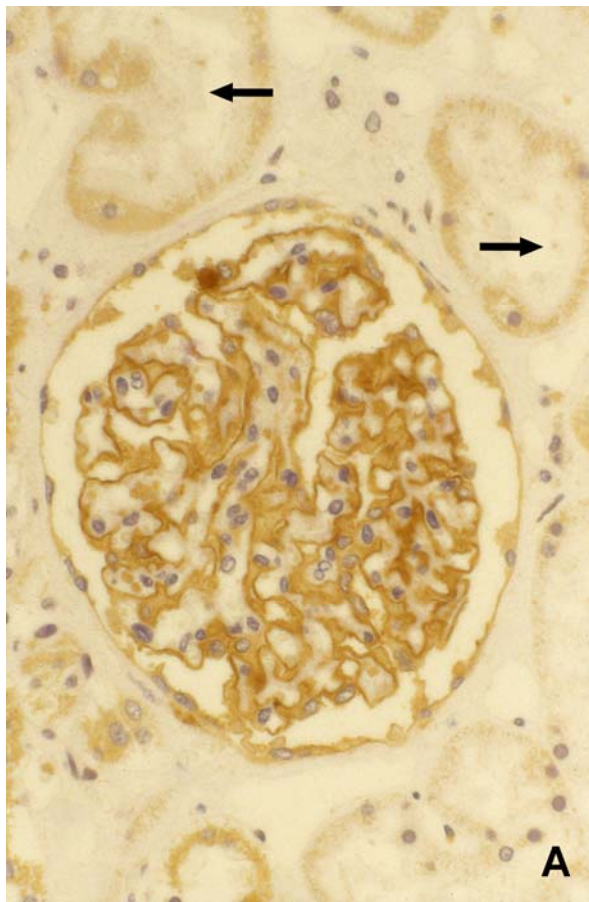
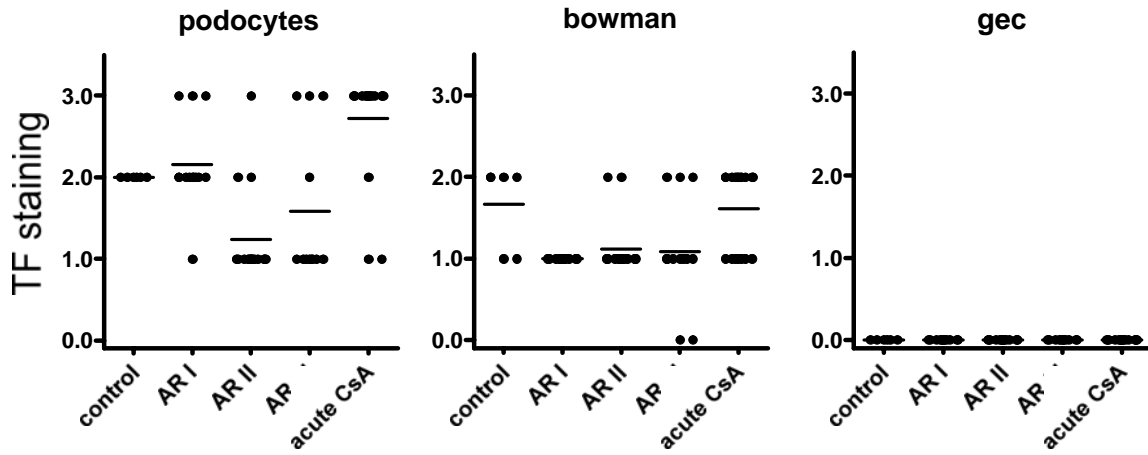
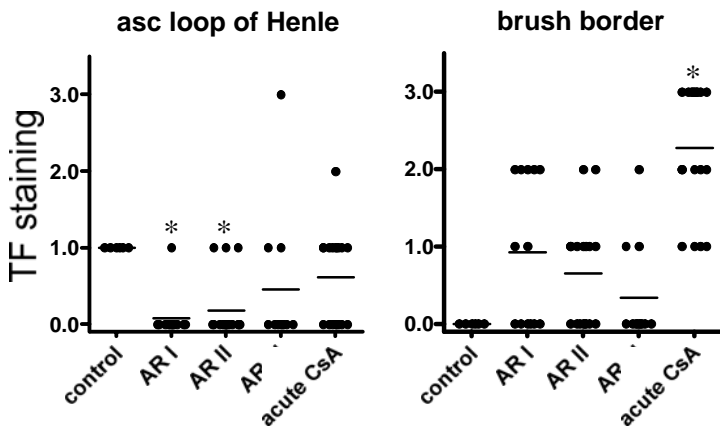


Fig. 3

a)



b)



c)

