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## **Dog as model for down-expression of E-cadherin and $\beta$ -catenin in tubular epithelial cells in renal fibrosis**

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### **Abstract**

Mechanism of renal fibrosis leading to end stage kidney remains still a challenge of interest in humans. The pathogenesis of chronic kidney disease is characterized by

progressive loss of kidney function and fibrosis. The mechanism of epithelial–mesenchymal transition (EMT) has been predominantly studied in in vitro studies, and we previously demonstrated the EMT of tubular epithelial cells in dogs. In this study, we examined and quantified the modifications of cadherin–catenin complex by immunohistochemistry of E-cadherin and  $\beta$ -catenin and the mesenchymal marker vimentin in 25 dogs with three different spontaneous inflammatory renal diseases. Results showed a significant down-expression of levels of E-cadherin and  $\beta$ -catenin directly correlated with the tubular–interstitial damage (TID). In TID grades 2 and 3, E-cadherin expression was significantly reduced ( $p < 0.001$ ).  $\beta$ -catenin expression was overall similar to E-cadherin. The mesenchymal associated protein, vimentin, was de novo identified in tubules within areas of inflammation. In this work, we identified the loss of cadherin or catenin expression as a progressive mechanism in tubulo-interstitial fibrosis, which allows dissociation of structural integrity of renal epithelia and loss of epithelial polarity. The dog might result more significant as model for new therapies.

Keywords Adhesion molecule, Canine, Fibrosis

## Introduction

By definition, epithelial–mesenchymal transition (EMT) is a phenotypic conversion of epithelial cells, leading to the loss of epithelial cell–cell–basement membrane contacts, structural–functional polarity, and acquisition of a fibroblastic phenotype [23]. A number of steps appear necessary to complete the process of EMT. Loss of the epithelial phenotype and disruption of adhesion molecules is the first phase of EMT, reported in several types of pathological processes: tumors, embryogenesis, and organ fibrosis [2, 15, 16, 21, 22]. Emerging evidence has established EMT as one of the major mechanism of renal fibrosis in human and other species [14]. In the latest studies, the authors showed that tubulo-interstitial damage (TID) is characterized by loss of differentiated epithelial cells and activation of mesenchymal cells (renal fibroblasts) leading to renal fibrosis [27]. Tubular epithelial cells (TECs) undergo phenotypic change as demonstrated by de novo vimentin expression and loss of cytokeratin during chronic renal disease. While, under normal circumstances, the TECs are attached to each other and to the basement membrane through specialized junctional complexes that include molecules such as cadherin and

catenin, during the injury, epithelial cells lose the polarity and the mechanisms of adhesion [18, 20]. These processes of cellular injury and detachment lead to the release of cytokines, different inflammatory mediators, and the recruitment of leukocytes to the site of injury. These inflammatory processes may both contribute to the injury and play a role in the repair mechanism that follows the injury [6, 7].

Canine renal pathology represents a good model for the examination of changes in the tubulo-interstitial compartment. In renal biopsies, early histomorphological signs of interstitial fibrosis in dogs are associated with an unfavorable prognosis, even if routine clinical parameters still indicate a compensated kidney function. We have recently demonstrated the similarity of the renal canine model to human, showing that the number of TECs undergoing EMT features is associated with the degree of TID [1]. The aim of the current study was to examine in the same distribution of damage the regulation of adherens junction complex (E-cadherin and  $\beta$ -catenin) in TECs. Numerous recent studies have shown that cellular adhesion molecules and their associated proteins may be critical early target in renal fibrosis murine model and in human transplanted kidney [2, 20]. To study the origin of the EMT, we focused our attention on the potential mechanisms of TECs activation in dogs with different types of spontaneous glomerular disease and associated tubulo-interstitial damage. We postulated that E-cadherin and  $\beta$ -catenin are key molecules in tubular EMT, and loss of expression of both markers is relevant in development of kidney fibrosis, through tubular EMT.

## **Materials and methods**

We collected open renal specimens from 25 dogs who were examined and diagnosed with renal disease in the Department of Pathology, University of Turin in 2007. Disease categories were based on histological, immunofluorescent, and ultrastructural examination, and the diagnosis were given using the criteria of the WHO classification of glomerular diseases. The following groups were included in this study: membranous glomerulonephritis (6), membranoproliferative glomerulonephritis (13), and minimal change disease (6). Clinical parameters (plasma creatinine and proteinuria) were obtained at the time of collection (see Table 1).

### Histological examination

Renal samples were fixed in 10% neutral buffered formalin, and sections were cut at 3  $\mu\text{m}$  in thickness and routinely stained. On Masson's Trichrome staining, interstitial fibrosis and inflammation were assessed at  $\times 200$  magnification and scored as follow: normal tubulo-interstitium (TID grade=0), mild tubular atrophy, and interstitial edema or fibrosis affecting up to 25% of the field of view (TID grade=1); moderate tubulo-interstitial fibrosis affecting 25–50% of a given field (TID grade=2); severe tubulo-interstitial fibrosis >50% of a field (TID grade=3).

#### Immunofluorescence

For immunofluorescence, unfixed renal tissue was OCT embedded, snap-frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Fluorescein Isothiocyanate-labeled anti-goat IgA, IgG, IgM, and complement C3 antibodies specific for the dog (Bethyl Laboratories INC., Montgomery, AL, USA) were used. Primary antibodies were omitted as negative controls.

#### Immunohistochemistry

An immunohistochemical panel was performed to assess the changes to the TECs. The following antibodies were used in this work: antihuman E-cadherin (clone 36, Transduction Laboratories, Lexington, KY, USA), antimouse  $\beta$ -catenin (clone 14, Transduction Laboratories) and anti vimentin (Dako, Glostrup, Denmark). For the immunostaining, paraffin kidney sections (4  $\mu\text{m}$ ) were dewaxed and rehydrated, and antigen retrieval was performed by microwave exposure for 3 min (three times) in a citrate buffer (pH 6.0) at 600 W. When the temperature of the buffer reached room temperature, the slides were rinsed in phosphate-buffered saline (PBS) and incubated with hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) 3% in methanol for 15 min to block endogenous peroxidase activity. After rinsing in PBS, background staining was blocked using PBS added of 10% bovine serum albumin (BSA, Sigma–Aldrich, St. Louis, MO, USA) for 20 min. The sections were then incubated with the primary antibody diluted in PBS 10% BSA for 60 min. Anti E-cadherin and anti  $\beta$ -catenin were diluted 1:70, anti vimentin 1:50 in PBS 10% BSA. After two washings with PBS, the sections were incubated with the secondary antibody provided by a commercial kit (Real EnVision™ peroxidase Detection System, Dako) for 30 min then washed twice in PBS; the reaction was developed with the DAB solution provided by the kit, washed in tap water, and counterstained with hematoxylin and PAS. Normal mammary gland was also used as positive control of the immune reaction for E-cadherin and  $\beta$ -catenin. We tested normal canine kidney tissue to quantify the expression of E-cadherin and  $\beta$ -catenin in TECs, and

we used normal mammary gland for comparison. Expression of both markers in control dogs results to be mostly total in cytoplasm and membrane of TECs.

#### Double immunohistochemistry

Vimentin and  $\beta$ -catenin double antigen-immunoperoxidase labeling was performed using a commercial kit (Vector Laboratories, Burlingame, CA, USA), according to manufacturer's instructions. Peroxidase activity was demonstrated firstly by DAB + Ni<sup>2+</sup> (grey to black chromogen reaction) for Vimentin and secondly by 3-amino-9-ethylcarbazole (AEC, red chromogen reaction) for  $\beta$ -catenin.

#### Quantification of immunohistochemistry and statistical analysis

Immunohistochemical staining was assessed with respect to its localization and its intensity. E-cadherin and  $\beta$ -catenin immunoreactivity was classified as membranous (localized to cell-cell boundaries) or cytoplasmic (uniformly distributed through the cytoplasm, with no recognizable distinction between membrane and cytoplasm). Quantification of E-cadherin and  $\beta$ -catenin expression in each case was graded according to loss in TEC of immune reaction for the two markers. Loss of cytoplasmic staining of adhesion molecules (E-cadherin and  $\beta$ -catenin) was assessed by counting the percentage of stain-negative cells per HPFs.

For each case examined, analysis was performed by counting ten fields ( $\times 200$ ), focusing on TECs. Expression of cytoplasmic staining for mesenchymal marker (vimentin) was evaluated by counting the percentage of positively stained TEC per HPFs. The staining intensity, based on the area of distribution, was also assigned from 0% to 100%.

The semiquantitative evaluation of immunostaining was evaluated by an electronic image analysis system (Adobe Photoshop CS3). Images were digitalized using a video camera connected to a single microscope and to a computer equipped with a frame grabber (Neotech Ltd, Eastleigh Hampshire, UK). In order to detect a possible relationship between the two percentages of the variables (E-cadherin or  $\beta$ -catenin and area), a scatter plot was used, grouping their values according to the nominal variable TID. Furthermore, the product of the percentage of the values for the intensity and the area was used to increase the sensitivity of the data.

Data are presented as means  $\pm$  SD. Analysis of variance (ANOVA) was performed, and the six comparisons among the mean values of the pairs were performed using the

Bonferroni Multiple Comparison Test. Statistical analyses were performed with GraphPad-InStat software (GraphPad Software, San Diego, CA, USA).

## Results

Tubulo-interstitial damage was a common finding in membranous and membranoproliferative glomerulonephritis (Fig. 1). In minimal change disease, only minimal areas of inflammation and fibrosis were detected (TID grade 1). All three grades of TID were present among the samples examined. For each case, the quantitative analysis data corresponding to the grade of TID are summarized in Table 2.

Renal samples, independent of the diagnosis, showed a variable intensity of positive immunostaining in the TECs for E-cadherin and  $\beta$ -catenin.

### Expression of E-cadherin

Uniform, strong membranous, and cytoplasmatic E-cadherin staining was localized in TECs in the controls ( $87.7\pm 2.0$  product, see Fig. 2a). In TID grade 1, E-cadherin immunoreactivity was minimally reduced when compared to normal TECs ( $62.2\pm 2.5$  product). In TID grades 2 ( $33.7\pm 2.0$ ) and 3 ( $12.5\pm 4.0$ , see Fig. 2b and c), E-cadherin expression was significantly reduced. Distribution in TECs, independently of the disease, was heterogeneous or negative compared with that in normal tubular epithelium and was characterized by patterns with variable degrees of membrane and cytoplasmic staining. This expression was preserved in the intact tubules next to the areas of inflammation. Table 1 summarizes the relationship between E-cadherin expression and the TID grade. E-cadherin expression in all cases examined significantly correlated with the histological grade ( $p < 0.001$ ).

### Expression of $\beta$ -catenin

Normal TECs showed expression of  $\beta$ -catenin; the signal was recorded not only more intensely at cell junctions, but also diffusely in the cytoplasm ( $92.5\pm 2.0$  product, see Fig. 2d). The  $\beta$ -catenin expression was similar to E-cadherin in all lesions examined. Expression of  $\beta$ -catenin was reduced in TID grade 1 ( $58.8\pm 2.2$  product, see Fig. 2e) compared to normal TECs ( $p < 0.001$ ). Scattered positive TECs in the TID grade 3 showed an exclusively granular cytoplasmic pattern (see Fig. 2f). Tubules next to the inflammation retained the cytoplasmic and membrane localization of catenin. Nuclear staining was also observed in some cells. Data are shown in Table 1.



### Expression of vimentin

No tubular expression of vimentin was detected in control dogs (Figs. 3, 4). De novo expression of vimentin marker was identified in tubular epithelial cells in different proportion, statistically correlated with the TID grade ( $p < 0.01$ ). More intense vimentin expression was restricted to areas of tubular damage, and concomitant expression of vimentin and  $\beta$ -catenin was recorded in the same tubular cross sections in double immunohistochemistry (see Fig. 5a and b). However, the expression of vimentin marker was demonstrated in TECs of degenerate atrophic tubules within areas of inflammation ( $48.2 \pm 10.3$  TECs/field in TID grade 3). Both intact tubules closed to the inflammation showed vimentin staining. Quantification of the percentage of tubules expressing vimentin marker is shown in Table 1.

### Examination of PAS-vimentin immunohistochemistry

showed a thickening of tubular basement membrane correlated with tubules vimentin positive; no ruptures were identified (see Fig. 6).

### Statistical results

The scatter plot used to detect the relationship between the two variables percentages of E-cadherin or  $\beta$ -catenin, and area showed that the four clusters of TID were homogeneous and evidenced a systematic difference between them (see Figs. 3 and 4). Moreover, the two variables were strongly correlated; so, a new variable, the product of the mentioned ones, was considered for the statistical analysis. ANOVA revealed that all the differences were highly significant ( $p < 0.001$ ). Therefore, the six comparisons among the mean values of the pairs were performed using the Bonferroni Multiple Comparison Test, and the differences were again highly significant ( $p < 0.001$ ).

The last analysis was the modelization of the composed variable product with respect to TID according to a linear model. Both the line plot and the statistical indices  $p$  value and  $R^2$  encourage to predict the variable product of E-cadherin or  $\beta$ -catenin and area according to a more suitable model, a quadratic one (see Figs. 3 and 4).

## Discussion

In this study, we sought to determine whether the EMT process was related to down-expression of E-cadherin and  $\beta$ -catenin and if this correlated with the different grades of TID in the most common spontaneous canine renal diseases.

The E-cadherin–catenin complex plays a fundamental role in the construction of epithelia. Cadherins comprise a family of calcium-dependent cell–cell adhesion proteins that play important role in embryonic development and maintenance of normal tissue architecture [5, 6, 28]. The cadherin–catenin complex binds to the cytoskeletal components, including actin bundles, and the linkage in this complex is crucial for E-cadherin to function normally [17].

The predominant epithelial isoform, E-cadherin, localizes in the lateral membrane of differentiated epithelia, providing the structural foundation for adherens junctions [18]. In fact, they not only function as static structural components of adherens junctions but they, along with some of their associated molecules, also play critical role in regulating cell-signaling pathways [3, 19, 26]. Likewise, the inflammatory processes may either contribute to the initial injury or occur as a consequence of the cellular injury involving specific changes in the expression and function of cell adhesion molecules that are necessary for the migration, attachment, and activation of leukocytes [26].

Cadherin–catenin complex alterations have been studied in the development of carcinogenesis model and to understand the mechanism of epithelial mesenchymal transition in the fibrotic process [2, 4]. Few papers demonstrate that changes in cadherin expression are fundamentals to the process of EMT and cellular motility [19, 24]. In the human adult kidney, the cadherins are differently expressed in various segments of the nephron. In a study of mouse kidney, Piepenhagen and Nelson [25] showed that E-cadherin is abundantly expressed in most segments of the nephron, including the proximal tubules. Cho and colleagues [8] also reported that E-cadherin is present in the proximal and distal tubules of newborn mice. In the present experiment, normal kidney from control dogs showed cytoplasmatic and membrane expression of both adhesion markers in all segments of the nephron.

The immunohistochemical profiles of E-cadherin and  $\beta$ -catenin observed in this study result not associated with the glomerular lesions; no correlation was found among the different classes of disease. On the contrary, the reduction in cadherin and catenin expression was strictly related to the TID: a statistical significant association ( $p < 0.01$ ) was found that correlated with the different evolution of TID.

Data on the expression of  $\beta$ -catenin in renal specimens are similar for E-cadherin with a similar frequency of alterations, and we also identified the presence on nuclear expression of catenin. Different in vitro studies have demonstrated that down-expression of E-cadherin is related with translocation of  $\beta$ -catenin from the membrane leading to the cytoplasm and then into the nucleus. This translocation suggests the loss of epithelial polarity and integrity [9].  $\beta$ -Catenin has a dual role in the EMT: it enhances cell–cell adhesion when bound to cadherin complexes in adherens junctions and also functions as a transcriptional co-activator upon entry into the nucleus [7, 11, 13]. The results obtained in this experiment evoke an interesting speculation: downexpression of cadherin–catenin system in TECs is a necessary step for transdifferentiation of these cells into cells acquiring mesenchymal features. The entire process requires alterations in morphology, cellular architecture, and adhesion capacity [10, 12, 31]. TECs under normal conditions are tightly connected to each other to form an integrated epithelial sheet through cell adhesion mechanisms fundamental for their functions, and no mesenchymal features are evident in TECs (vimentin expression in control dogs:  $0.0\pm 0.0$ ). As disease progresses, epithelial tubular markers largely disappeared, and coexpression of vimentin and epithelial markers in TECs becomes evident ( $\beta$ -catenin expression in T1D grade 3:  $12.0\pm 4.4$ ). Positivity for mesenchymal antigen and loss of adhesion junction properties were mainly detected in well-preserved tubular structures without signs of tubular basement membrane disruption or possible cell migration into the interstitium, as demonstrated by PAS counterstain.

To investigate this mechanism, we performed double immunohistochemistry for vimentin and  $\beta$ -catenin. This technique permits to identify the colocalization of both markers and define the process as step by step due to the progressive modification in the same tubular cross section.

This work confirms that loss of cadherin or catenin expression is an early mechanism in tubulo-interstitial fibrosis, which allows dissociation of structural integrity of renal epithelia and loss of epithelial polarity. To enable migration of single epithelial cells, the strong cell–cell adhesion, a feature of epithelial cells, has to be reduced. Therefore, the reduction of cellular adhesion is associated with epithelial dedifferentiation mechanism. EMT is easily started by a combination of cytokines associated with proteolytic digestion of basement membranes upon which epithelia reside [10, 29, 30]. The canine renal model offers different advantages in the study for the role of adhesion molecules and the EMT mechanism in kidney, such as the development of spontaneous diseases, the slow

progression of the disease, and overall, the similarity of inflammatory lesions in the canine kidney to humans.

In summary, we could describe the process of EMT in different events involving: loss of adhesion properties (loss of catenin and cadherin expression) and de novo expression of mesenchymal features (vimentin positive reaction in TECs). This study provides the first evidence that TECs can undergo phenotypic change toward a mesenchymal expression during progressive renal fibrosis in canine glomerulonephritis on the basis of de novo vimentin expression and down-expression of epithelial markers of junction. While down-regulation of cadherins is well described in cultured cells and murine model, it still remains to be shown whether such events occur in spontaneous renal pathology in human [28, 29, 32]. On the base of this experimental study, the dog becomes an important model for further investigations, especially for the expression of TGF- $\beta$ , a major regulator of EMT and cadherins expression in the TECs.

**Table 1** Summary of clinical results of dogs

TID grade	Plasma creatinine (mg/dl)	UPC <sup>a</sup> value
0	1.1 $\pm$ 0.2	<0.2
1	1.8 $\pm$ 0.4	0.3 $\pm$ 0.04
2	3.8 $\pm$ 1 2	0.7 $\pm$ 0.14
3	5.3 $\pm$ 2.5	0.9 $\pm$ 0.24

<sup>a</sup> Urine protein/creatinine ratio

**Table 2** Summary of immunohistochemical results

TID grade	0 (5 dogs)	1 (11 dogs)	2 (7 dogs)	3 (7 dogs)	<i>p</i> Value
E-cadherin	87.7 $\pm$ 2.0	62.2 $\pm$ 2.5	33.7 $\pm$ 2.0	12.5 $\pm$ 4.0	<0.0001
$\beta$ -catenin	92.5 $\pm$ 2.0	58.8 $\pm$ 2.2	31.5 $\pm$ 3.0	12.0 $\pm$ 4.4	<0.0001
Vimentin	0.0 $\pm$ 0.0	10.2 $\pm$ 2.1	26.3 $\pm$ 5.2	48.2 $\pm$ 10.3	<0.0001

Results are reported as means (obtained from the multiplication of the values for the intensity and the percentage of positive tubular epithelial cells)  $\pm$  SD

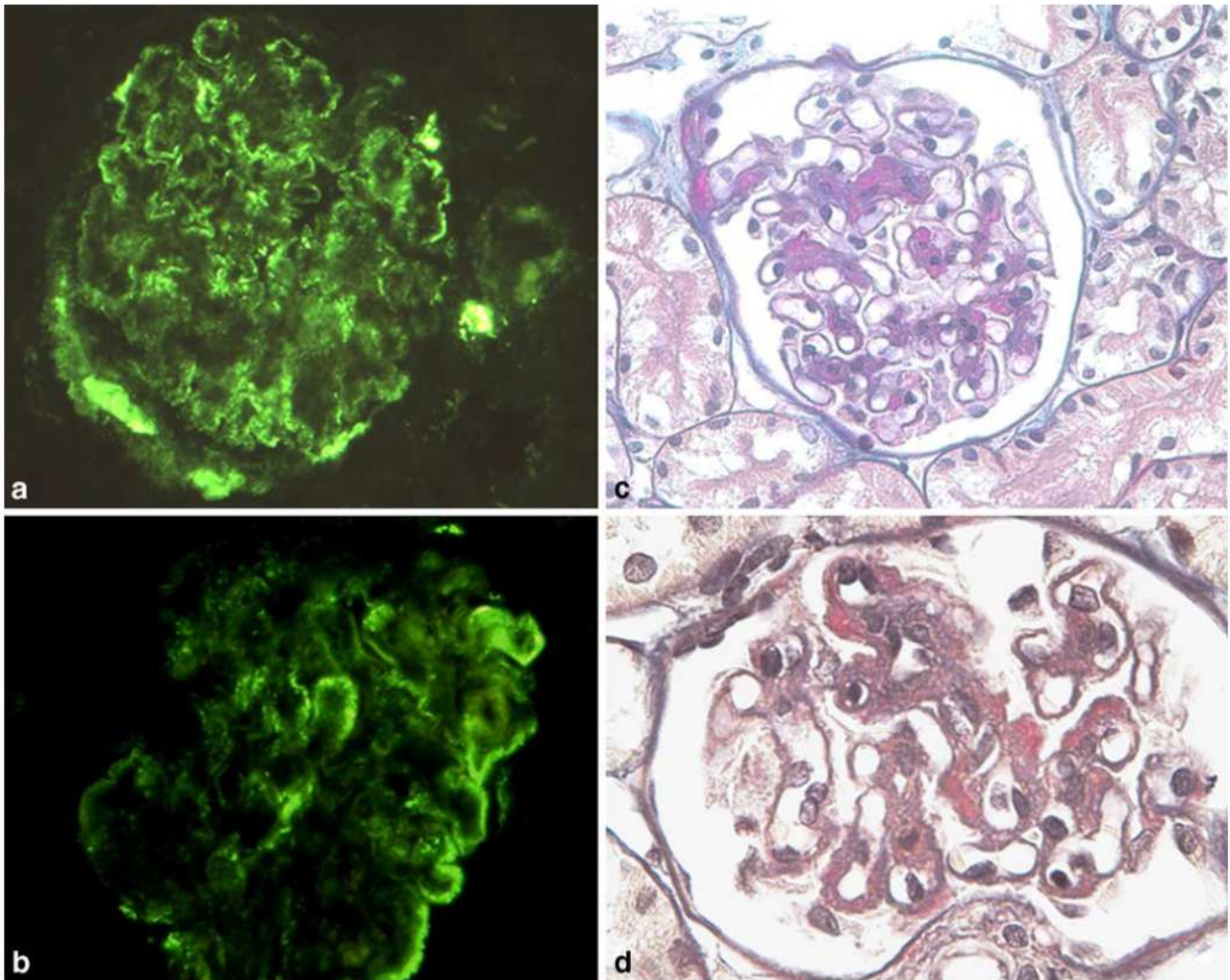


Fig. 1 a Granular pattern deposition along the glomerular basement membrane associated with mesangial distribution. (FITC-anti-goat IgG,  $\times 400$ ). b Diffuse, intense granular deposits along the glomerular basement membrane. (FITC-anti-goat IgG,  $\times 400$ ). c Deposition along the basement membrane and in the mesangium in a membranoproliferative glomerulonephritis (AFOG,  $\times 400$ ). d Diffuse thickening of glomerular basement membrane due to intramembranous deposition in a membranous glomerulonephritis (AFOG,  $\times 400$ )



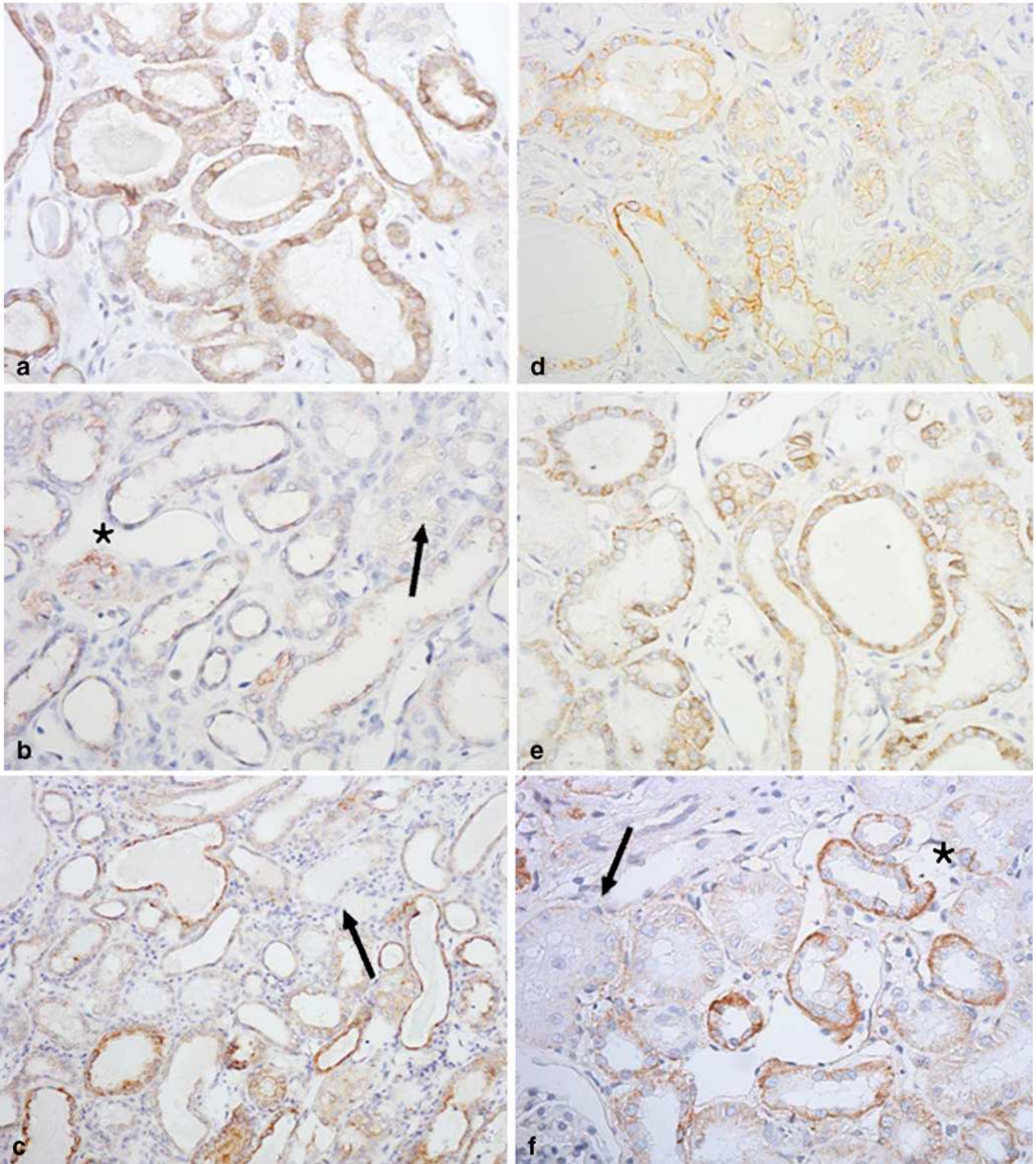


Fig. 2 a Normal E-cadherin cytoplasm and membrane expression in control kidney (×200). b Progressive lost of cadherin expression in tubular epithelial cells (arrow) and tubules with normal expression (asterisk) in TID grade 2 (×400). c Negative staining of E-cadherin in multifocal tubules (arrow) in TID grade 3 (×100). d  $\beta$ -catenin intensely stained membrane of epithelial tubular cells in control kidney (×200). e Progressive lost of  $\beta$ -catenin expression in scattered epithelial cells in TID grade 1 (×200). f Lost of  $\beta$ -catenin expression evident in tubular cross sections (arrow) and tubules with normal expression (asterisk) in TID grade 3 (×200)

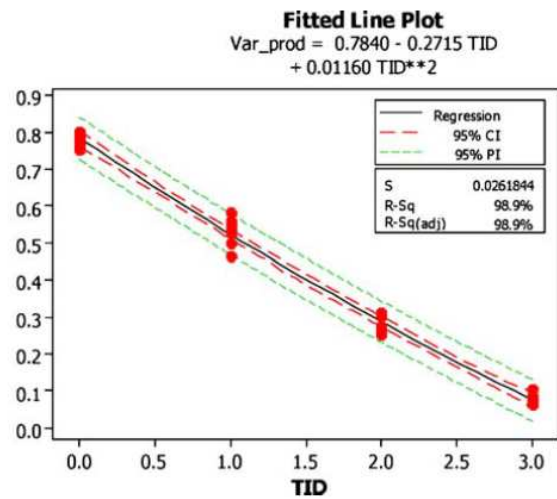
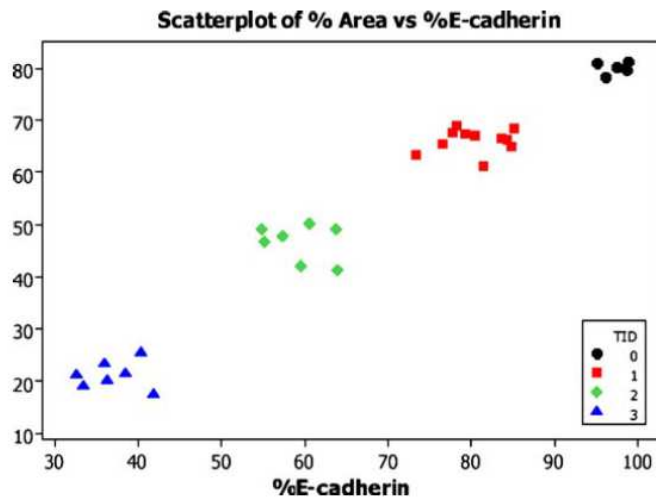


Fig. 3 Left scatter plot to detect the relationship between the two variables percentages of E-cadherin and area. Right modelization of the composed variable product for E-cadherin with respect to TID

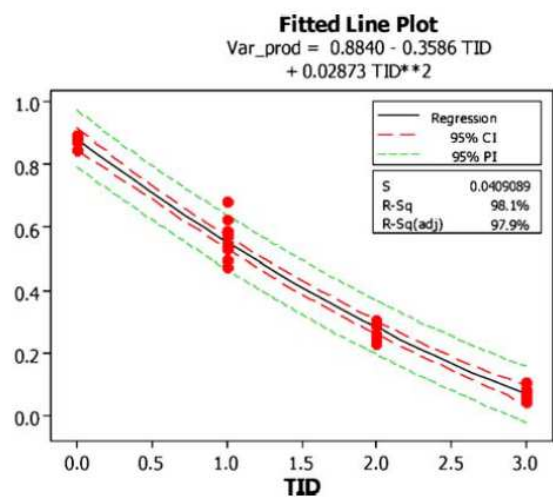
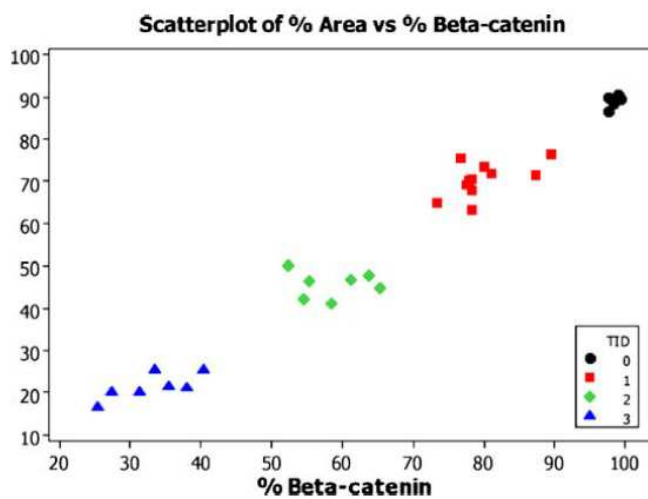


Fig. 4 Left scatter plot to detect the relationship between the two variables percentages of  $\beta$ -catenin and Area. Right modelization of the composed variable product for  $\beta$ -catenin with respect to TID



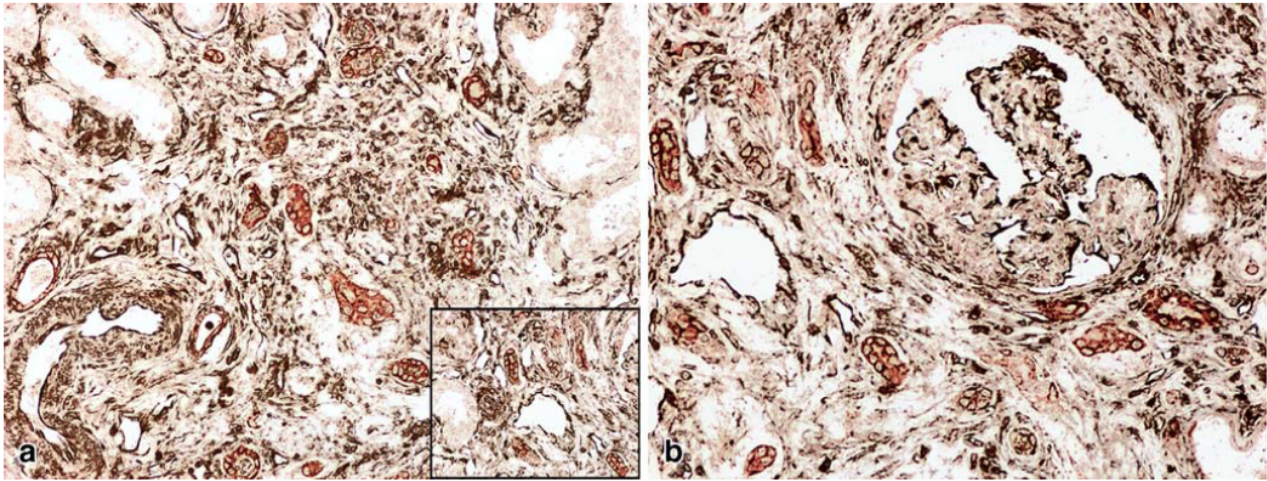


Fig. 5 a, b Scattered epithelial tubular cells  $\beta$ -catenin positive stained in red and several epithelial tubular cells vimentin positive stained in black in same tubular cross section (arrow) in T1D grade 2 (double immunohistochemistry, a  $\times 400$ , b  $\times 600$ )

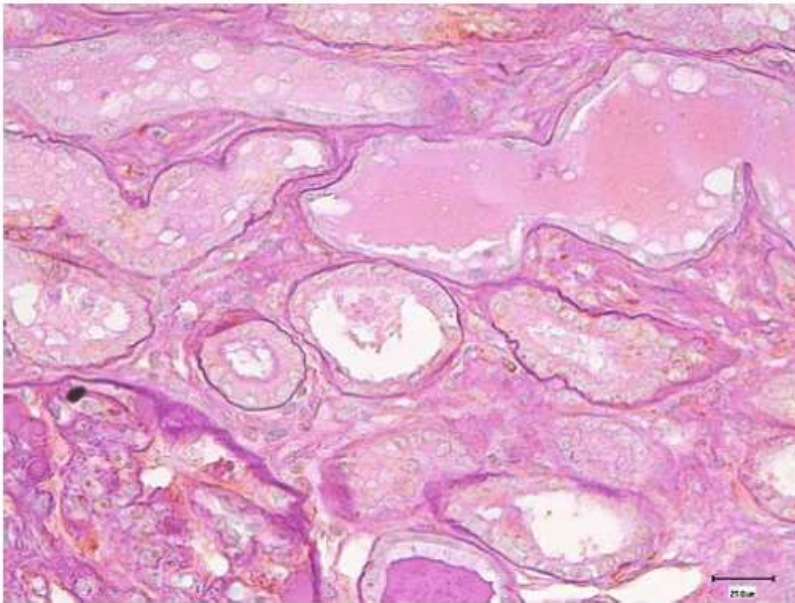


Fig. 6 Down-expression of  $\beta$ -catenin associated with tubular basement membrane thickening, no sign of rupture (Vimentin-PAS counterstained,  $\times 200$ )

Conflict of interest statement We declare that we have no conflict of interest.

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