Proliferation Signal Inhibitor-associated proteinuria in a renal transplant recipient: Dysfunction of proximal tubular epithelial cells is a result of decreased cubilin and/or megalin expression?

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Thank you to my family, my mother, father, brother and my friends for their support.

Sincerely
Myakala. Komuraiah
Abstract

Background The proliferation signal inhibitors (PSIs) sirolimus (SRL) and everolimus (ERL) are the potent immunosuppressive drugs using in organ transplantation and has been used successfully in renal transplant recipients (RTX) as well. PSIs are the key factors to overcome the allograft rejections after successful organ transplantation since the immune system starts to react against the graft. SRL and ERL prevents the action of immune system b inhibits the proliferation of T- and B-cells by inhibiting the intracellular signaling of interleukin-2. The presence of excess amount of serum proteins including albumin in the urine is considered as proteinuria, which reflects the loss of kidney function. The occurrence of proteinuria can be the result of abnormal glomerular filtration and/or impaired tubular endocytic function of renal proximal tubular epithelial cells (PTECs). Megalin and cubulin are two scavenger receptors present on epical surface of PTECs and involved in reabsorption of proteins after glomerular ultrafiltration process in the kidney. Proteinuria appears too high in renal transplanted patients during ongoing treatment with PSIs.

Aim Our study aimed to investigate and correlate the expression level of megalin and cubulin and albumin uptake in PTEC of renal transplanted patients before and after conversion to PSI.

Methods To retrieve the maximal expression of our interest molecules in renal PTECs, we optimized antigen retrieval (AR) method and primary antibody dilution for each molecule separately. An optimization experiment was performed on 3 different normal patients renal biopsies were used. Later, human renal biopsy specimens originated from 4 different renal transplanted patients were used in this study. From all the 4 patients biopsy specimens were taken before and ongoing administration of PSIs (SRL, ERL). The expression of megalin, cubulin and albumin uptake in PTEC of renal transplant patients was determined by immunohistochemical staining.

Results Based on the optimization experiments, we selected the AR method and primary antibody dilution for the expression of megalin, cubulin and albumin uptake. In 4 renal transplanted patients following administration of PSIs results in patients 1, 2, 3 expression of megalin, cubulin and albumin uptake during ongoing PSI treatment was not comparable or even more intense than before PSIs introduction. The expression of megalin, cubulin and albumin uptake was reduced in patient 4 during ongoing PSI treatment.

Conclusion Our findings suggest that the renal transplant patient 4 developed proteinuria during PSI medication. The expression of megalin, cubulin and albumin uptake was markedly decreased during ongoing PSI treatment in patient 4. We concluded that there is a direct link between PSI medication and tubular dysfunction, which might cause proteinuria.
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**Abbreviations**

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<th>Description</th>
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<tbody>
<tr>
<td>AR</td>
<td>Antigen retrieval</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin II type 1 receptor</td>
</tr>
<tr>
<td>ACE-inh</td>
<td>Angiotensin converting enzyme inhibitors</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>CNI</td>
<td>Calcineurin inhibitor</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>ERL</td>
<td>Everolimus</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed Paraffin embedded</td>
</tr>
<tr>
<td>kD</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>PTEC</td>
<td>Proximal tubular epithelial cells</td>
</tr>
<tr>
<td>PSI</td>
<td>Proliferation signal inhibitor</td>
</tr>
<tr>
<td>SRL</td>
<td>Sirolimus/Rapamycin</td>
</tr>
</tbody>
</table>
1. Background

1.1 Renal physiology

Under normal physiological conditions of kidney, blood filtration takes place through the glomerular barrier and glomerular ultrafiltrate contains proteins, charged molecules (ions), hormones, salts and drugs. The primary urine enters into the renal tubule where reabsorption and excretion of electrolytes and proteins takes place by different mechanisms. Finally, the urine is collected in the renal pelvis. The reabsorption of proteins including albumin takes place in the renal proximal tubular lumen. Besides other proteins, albumin is preferentially reabsorbed by receptor-mediated endocytosis in the proximal tubule because of its size and charge. The reabsorption of albumin and other proteins were initiated by binding to the multiligand receptors megalin and cubilin (1, 2, 3). In proximal tubular epithelial cells (PTEC), after receptor mediated endocytosis proteins were further processed to lysosomes for degradation and later receptors recycle onto the apical membrane of PTEC (23, 30).

![Figure 1](image_url) shows the structure and function of the nephron in the kidney

1.2. Proteinuria

The presence of excess or abnormal amount of serum proteins in the urine is known as proteinuria. The serum proteins were considered as albumin because albumin is the most abundant protein present in blood serum and it represents 60% of all plasma proteins. Albumin is involved in the regulation of oncotic pressure and also in the transport of hydrophobic molecules. The increase of albumin excretion is traditionally considered as loss of proteins that indicates functional loss of kidney or chronic kidney disease. Proteinuria can be the result of abnormal glomerular filtration process and/or impaired endocytic function of PTEC. The abnormal glomerular filtration might be the reason of increased permeability of glomerular capillary wall. Proteinuria is associated with many diseases such as diabetis, hypertension, immune disorders, IgA nephropathy and can be observed after organ transplantation as well.
1.2.1. Megalin receptor

The endocytic transmembrane protein megalin (Figure 2) (600 kDa) belongs to the low-density lipoprotein (LDL) type receptor family (4). Megalin was identified by Kerjaschki and Farquhar identified as a target antigen in rat model of heymann nephritis (5, 6). Megalin is expressed and co-localized with cubilin in renal PTEC, visceral yolk sac, small intestine and cytrophoblast of the placenta (reviewed in 7). In addition, megalin was observed in ependymal cells, thyroid cells, oviduct, type-2 pneumocytes, parathyroid secreting cells, epididymis, choroid plexus, the endometrium, ciliary epithelium of the eye (reviewed in 7, 8), embryonic tissues of trophoectodermal cells and neuroectoderm (9, 10). Numerous ligands of megalin are known, which includes vitamin carrier proteins, albumin, myoglobin, lactoferrin, lipoproteins, hormones, enzymes and drugs. The expression of megalin depends upon receptor-associated protein (RAP) (11). RAP is a chaperon or escort protein and can function as receptor antagonist (13, 14). RAP protects the newly synthesized receptor during the transport to the cell surface, because it prevents the early association of megalin with its ligand (15, 16). RAP binds to other LDL-receptor family members as well and its role in receptor folding is very likely (12, 13). The endocytic function of PTEC is regulated by different factors, which has conceivable impact on the membrane expression of endocytic receptors. These factors include, angiotensin II (Ang II), insulin and transforming growth factor β (TGF-β). L.M. Russo et al demonstrated in diabetic nephropathy in experimental animal studies that TGF-β might play an important role in the down regulation of megalin expression (62). Cultured PTEC treated with insulin or with glucose in high concentration (17.5 mM) showed increased expression of megalin, whereas Ang II resulted in decreased megalin expression (63). This study suggests that the megalin expression is regulated by competitive cross talk between Ang II type1A receptor and insulin-mediated signaling pathways (63). Nevertheless, how the crosstalk between Ang II and insulin regulates megalin expression, is still unknown.

Figure 2 Schematic diagram (reprinted from (24)) shows expression of megalin and cubilin in PTEC where they play a central role in the reabsorption of proteins, vitamins and lipids through endocytosis. Megalin and cubilin bind a number of ligands with their binding domains (complement-type repeats and CUB domains, respectively).
1.2.2. Cubilin receptor

Cubilin (Figure 2) is a peripheral membrane protein (460 kDa), which is expressed on the apical surface of renal PTECs, visceral yolk sac and co-localized with megalin because it lacks transmembrane and intracellular segments. Previously cubilin is known as intrinsic factor-vitamin B$_{12}$ receptor as well. Cubilin in small intestine does not have structural homology with other endocytic receptors (17, 18). Hereditary megablastic anemia 1 or Imerslund-Grasbeck syndrome are caused by gene defects of cubilin. These patients suffer from vitamin B$_{12}$ malabsorption and proteinuria (35). In addition, cubilin was found in small intestine and thymus as well (19) and also in lysosomes (17). In rats, both megalin and cubilin expression was observed in glomerular podocytes (20). Similarly to megalin cubilin has numerous ligands. Some of the ligands bind to both receptors and some of them show specific interaction with their receptors (reviewed in 23, 24). The ligands for cubilin include intrinsic factor-vitamin B$_{12}$, vitamin D binding protein, albumin, myoglobin, hemoglobin, transferring, apolipoprotein A1 and high-density lipoprotein. It is known that cubilin interacts with RAP at sub cellular level (26, 27) but the posttranslational proceeding or distribution of this receptor still remained unknown. The endocytic function of cubilin is activated when it interacts with megalin (55, 60)

1.2.3. Role of megalin and cubilin in renal proximal tubule

Under normal kidney function, intermediate and low molecular weight proteins pass through the glomerulus barrier. The filtered proteins are reabsorbed in the proximal tubule that is connected to the Bowman’s capsule. In renal PTECs, megalin and cubilin mediate the reabsorption of proteins (albumin, carrier bound proteins, vitamins), which are present in the glomerular ultrafilterate (2, 25). Experimental studies suggest that, megalin binds directly to its ligand and internalizes it for lysosomal digestion but the cubilin-ligand complexes require megalin for further process (28, 29). The ligand–receptor interaction is Ca$^{2+}$ dependent (1, 28). It was clearly demonstrated that megalin deficient mice show increased excretion of albumin in the urine, as a result of defective tubular reabsorption (31, 32). Dogs with cubilin deficiency show dysfunctional tubular reabsorption also (33). Patients with hereditary malabsorption of vitamin B$_{12}$ often suffer from proteinuria due to structurally abnormal expression of cubilin (34, 35).

![Figure 3](image-url) Figure 3 Schematic diagram (reprinted from (29)) demonstrates the megalin/cubilin-mediated endocytosis in renal proximal tubule.
Ligands are internalized through the apical clathrin-coated pits in intermicrovillar areas (IMVA) into coated vesicles (CV) to form endosomes. The transfer of ligands to lysosomal degradation occurs through endosomal compartments (E). Then, the receptors are returned to apical plasma membrane through dense apical tubules (DAT).

### 1.3. Immunosuppressive drugs and their mechanism of action

Immunosuppressive drugs are key factors to overcome the allograft rejections after successful organ transplantation since the immune system starts to react against the graft. These drugs can be divided into 5 groups according to their mechanism of action (Figure 4), which includes calcineurin inhibitors (cyclosporine A, tacrolimus), mTOR inhibitors (sirolimus, everolimus), metabolic toxins (methotrexate), antibodies (anti CD3 mAb) or anti-inflammatory agents (dexamethasone). Immunosuppressive agents prevent the activation of the immune system by inhibiting the activation of immune mediated cells through different mechanisms. Immunosuppressive drugs can cause adverse effects like hyperglycemia, peptic ulcers, dyslipidemia, liver and kidney damage and increase the risk for infections.

![Diagram](Figure.png)

**Figure 4:** Schematic diagram illustrates different immunosuppressive drugs and their role of action in immunosuppression (reprinted from (38)). mAb, monoclonal antibody; MHC, major histocompatibility complex; TCR, T-cell antigen receptor; MAP, mitogen-activated protein; IKK, IK-kinase; NFAT, nuclear factor of activated T cells; AP-1, activated protein 1; NF-κB, nuclear factor κB; PI-3, phosphatidylinositol 3-phosphate; mTOR, mammalian target of rapamycin; PSI, proliferation signal inhibitor; MMF, mycophenolate mofetil.

Proliferation signal inhibitors (PSIs) include sirolimus (SRL) and everolimus (ERL). PSIs are newly discovered potent immunosuppressive drugs and successfully used in organ (kidney, heart) transplantation (37, 38).

**1.3.1. Proliferation signal inhibitors (PSIs)**

Sirolimus is a potent immunosuppressive drug using in organ transplantation and it has been used successfully in renal transplant recipients (RTX) as well (39). SRL was originally isolated from *Streptomyces hygroscopicus* Chemically, SRL is a macrocyclic lactone. SRL inhibits the
proliferation of T- and B-cells by inhibiting the intracellular signaling of interleukin-2. SRL binds to the cytoplasmic protein FK-binding protein 12 which blocks mammalian target of rapamycin (mTOR) pathway and consequently causes arrest of cell cycle in G1-S phase. Similarly to SRL, ERL is also an mTOR inhibitor and it is a derivative of SRL. ERL shows similar mode of action in the regulation of T-cell mediated immune response. SRL and ERL differ in their pharmacokinetic properties ERL has a longer half-life than SRL and the oral bioavailability of ERL is higher (40).

1.4. PSI associated proteinuria in renal transplant recipients

Proteinuria appears too high in number of patients, which reflects the renal dysfunction, is associated when immunosuppression was converted to mTOR inhibitors (46, 47, 48). The PSIs or mTOR inhibitors are mostly used in renal and heart transplantation until last few years in contrast to using calcineurin inhibitors (CNIs). Proteinuria occurrence reflects a progression of kidney dysfunction and can be a sign of chronic renal damage. In contrast to CNIs PSIs do not have nephrotoxic effects. Furthermore, PSIs have an antitumor activity. However, several clinical studies showed that, PSIs treatment is often associated with severe adverse side effects such as edema or proteinuria. Proteinuria can arise after conversion to PSI treatment or occur in case of de novo PSI treatment (41, 42, 43). A considerable increase in proteinuria was observed in long-term cardiac patients after switch to SRL after CNI based immunosuppression (41). It was demonstrated that renal transplant patients developed proteinuria after receiving PSI. Similar phenomenon was not observed in transplant patients treated with CNIs (45). The urinary protein loss in PSI-treated patients can be prevented by treatment with angiotensin converting enzyme inhibitors or angiotensin II receptor antagonists (57, 58, 59).

Proteinuria can be reverted after PSI withdrawal, which indicates a direct role of PSI in proteinuria (43, 49). In renal transplant recipient’s proteinuria was developed after conversion to mTOR inhibitors (51, 52) and proteinuria also has been observed in pediatric renal transplant recipients after SRL therapy (56). A previous case report suggested that, severe proteinuria was observed in a renal transplant patient during SRL treatment. The study indicates that proteinuria was the result of a decreased tubular protein reabsorption (49). So far, the explanation of reduced albumin reabsorption is not known. In contrast to tubular proteinuria, the occurrence of proteinuria might be infiltration of glomerular barrier also. It has been proved that proteinuria is associated with the hemodynamic changes of CNI withdrawal or preferably, the result of PSI induced glomerular or tubular damage (54). Contradictory evidences support both hypotheses. The tubular hypothesis suggested that, they did not find the albumin uptake in proteinuric transplanted patients in the presence of normal GFR (49). However, evidences show that post-transplantation glomerulonephritis, podocyte injury or SRL induced de novo focal segmental glomerulosclerosis can induce glomerular proteinuria. Nevertheless, the underlying mechanism is still remained unclear or controversial (44, 50).

In our previous in vitro study, we have found that direct administration of PSIs results in decreased albumin endocytosis and down-regulated cubilin and megalin expression in cultured PTEC cell line (HK-2 cells).
1.5. Aim of the study

Our retrospective study investigated the expression of megalin and cubilin and albumin uptake in PTEC of renal transplant patients before and after conversion to PSI. We aimed to investigate and correlate the expression level of cubilin/megalin to albumin reabsorption on PSI treated renal transplant recipients. Therefore, we examined by immunohistochemical staining on serial renal biopsies of transplant patients. We hypothesize that PSI medication might result in reduced expression of albumin receptors (cubilin, megalin) with the consequence of proteinuria occurrence. The study was performed in collaboration with the Department of Nephrology, University Hospital of Bern (Inselspital).

2. Materials and Methods

2.1. Patient data

Four patients with biopsies before PSI treatment and after introduction of PSI were enrolled in our study. Three patients were treated with ERL and 1 patient was treated with SRL. The Department of Nephrology, (University Hospital of Bern, Inselspital) provided us the demographic data (Table 1) and medical records (Table 2 and 3) of the patients.

<table>
<thead>
<tr>
<th>Date of birth</th>
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<th>Patient 2</th>
<th>22.5.1976</th>
<th>Patient 3</th>
<th>12.10.1941</th>
<th>Patient 4</th>
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<td>Female</td>
<td></td>
<td>Male</td>
<td></td>
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<td></td>
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<tr>
<td>PSI</td>
<td>ERL</td>
<td>ERL</td>
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<td>ERL</td>
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<tr>
<td>Original kidney disease</td>
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<td>MPG</td>
<td>Not known</td>
<td>ADPKD</td>
<td></td>
<td></td>
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<tr>
<td>Age of recipient at tx (years)</td>
<td>53.1</td>
<td>30.03</td>
<td>Not known</td>
<td>64.92</td>
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<td></td>
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<tr>
<td>Gender of donor</td>
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<td>Male</td>
<td>Not known</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Age of donor at Tx</td>
<td>42</td>
<td>62</td>
<td>Not known</td>
<td>65</td>
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<td>3</td>
<td>Not known</td>
<td>0</td>
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<td>HLA mismatches</td>
<td>4</td>
<td>3</td>
<td>Not known</td>
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<td></td>
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</tr>
</tbody>
</table>

Table 1 Demographic data of the patients. Abbreviations: TX: Transplantation; SLE: Systemic Lupus Erythematosus; HLA: Human Leukocyte Antigen; CMV: Cytomegalovirus; MPG: Membranous Proliferative Glomerulonephritis; ADPKD: Autosomal Dominant Polycystic Kidney Disease.
Table 2 shows medications. Abbreviations: CyA: Cyclosporin A; MMF: Mycophenolate Mofetil; ACE: Angiotensin-converting Enzyme.

Table 3 shows kidney functions of the patients. Abbreviations: GFR: Glomerular Filtration Rate.

2.2. Antibodies

The following primary antibodies were used in this study: rabbit anti-human LRP2 (megalin) (Sigma-Aldrich), goat anti-human cubilin (Santa Cruz Biotechnology), rabbit anti-human serum albumin (Gene Tex). The primary antibodies were detected with secondary anti-rabbit antibodies (HRP-labeled) (Dako EnVision™+ System). For the detection of anti-human cubilin, rabbit anti-goat antibodies were used as bridge antibodies between the primary and the secondary (HRP-labeled) antibodies.

2.3. Detection of cubilin, megalin and albumin by immunohistochemical (IHC) staining

The biopsy specimens were formalin fixed and paraffin embedded (FFPE) and analyzed according to Banff 07 criteria. The immunostaining was observed by using a light microscope (Leica DM-RB and Olympus DP 10 camera) with magnification length 40x. The sections were dewaxed prior to IHC staining in xylol bath (6 x 3 minutes) and rehydrated through alcohol gradient as follows, 2 x 3 minutes in EtOH (94%) and 1 x 3 minutes in EtOH (70%). This step was followed by antigen retrieval (AR) and IHC staining.

2.4. Optimization of antigen retrieval (AR) method
After rehydrate the sections in EtOH, to restore the immunoreactivity of a particular antigen, sections were pre-treated with different AR methods. The heat induced epitope retrieval (HIER) method and proteolytic enzyme induced epitope retrieval (PIER) method are the most common techniques to retrieve the epitopes. We performed HIER and PIER methods at different temperatures vary with time intervals (table 4 and 5). Different buffers were used for HIER method (table 4) and two different proteolytic enzymes for PIER (table 5).

<table>
<thead>
<tr>
<th>Buffers</th>
<th>Temperature and time</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate-monohydrate (0.01 M), pH 6 (pH was adjusted with NaOH, 6M)</td>
<td>Slides in pressure cooker</td>
<td>Citrate D</td>
</tr>
<tr>
<td>Citrate-monohydrate (0.01 M), pH 6 (pH was adjusted with NaOH, 6M)</td>
<td>Slides in microoven at 80°C for 30 minutes</td>
<td>Citrate at 80°C</td>
</tr>
<tr>
<td>Citrate-monohydrate (0.01 M), pH 6 (pH was adjusted with NaOH, 6M)</td>
<td>Sections in microoven at 550 W for 8 minutes after that at 125 W for 10 minutes</td>
<td>Citrate M</td>
</tr>
<tr>
<td>Tris base (0.1 M), urea (0.83 M), pH 9.5</td>
<td>Sections in microoven at 550 W for 8 minutes after that at 125 W for 10 minutes</td>
<td>Urea</td>
</tr>
<tr>
<td>EDTA (0.017 M), Tris base (0.02 M), tri-sodium citrate (0.01 M), pH 7.8</td>
<td>Section in microoven at 550 W for 8 minutes after that at 125 W for 10 minutes</td>
<td>TEC buffer</td>
</tr>
</tbody>
</table>

**Table 4:** different buffers of HIER methods vary in temperature and time

<table>
<thead>
<tr>
<th>Proteolytic enzyme</th>
<th>Temperature</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protease (100 mg) dissolved in 50 ml TBS buffer (Tris Hcl 50 mM, 150 mM NaCl)</td>
<td>Sections at 41°C for 6 minutes on heat block</td>
<td>Pronase</td>
</tr>
<tr>
<td>Trypsin (50 mg) dissolved in 50 ml buffer (calcium-chloride di hydrate 0.05 M; NaCl 1 M; tris hydroxy methane 0.12 M)</td>
<td>Sections at 37°C for 20 minutes in water bath</td>
<td>Trypsin</td>
</tr>
</tbody>
</table>

**Table 5:** different proteolytic enzymes for PIER methods varies in temperature and time

We tested all the 7 AR methods separately for megalin and cubilin expression and albumin detection on random kidney biopsies. The kidney biopsies originated from three patients.

**2.5. Optimization of primary antibody dilution**

Since the optimal antibody titer depends on the conservation of the biopsies, on the AR method and on the tissue type of the biopsy, the optimization of the primary antibody is crucial. We tested the primary antibody dilution using three dilution titers. For megalin, dilution titers included 1:30, 1:60 and 1:120, for cubilin 1:50, 1:100 and 1:200, for albumin staining 1:200, 1:400 and 1:800 respectively. The antibody was administered into each renal biopsy in 100 µl for 60 minutes. The antibody was diluted in antibody diluent from DAKO.
2.6. Immunohistochemical staining

After optimization of antigen retrieval method and primary antibody dilution, we performed the IHC staining on the renal biopsy sections. Deparaffinisation and rehydration was followed by AR method. For megalin, citrate buffer with pressure cooker (citrate D) was used as AR method and the primary antibody was diluted in 1:60. For cubilin, citrate at 80°C as AR method was used. The biopsy sections were incubated with the primary goat anti-human cubilin antibody diluted in 1:100. For albumin uptake citrate D as AR method was used and the primary rabbit anti-human serum albumin antibody was added in dilution of 1:400 with respective incubation time of 1 hour at room temperature. The primary antibodies were administered into each renal biopsy in 100 µl for 60 minutes at room temperature. After subsequent incubation with the primary anti-megalin and anti-albumin antibodies, sections were incubated for 1 hour at room temperature with the secondary anti-rabbit antibody conjugated with peroxidase. For the detection of cubilin, rabbit anti-goat antibody as bridge antibody was used in 1:500 dilution. All primary and secondary antibodies were diluted in “antibody diluent” from DAKO. As secondary antibody, Dako EnVision™+ System (HRP labeled) was added for 50 minutes and the sections were kept in dark. The staining was completed by a 3-10 minutes incubation with 3,3’-diaminobenzidine (DAB) + substrate-chromogen, which results in brown-colored precipitate at the antigen site. Later, biopsy sections were concisely counterstained with haematoxylin and mounted. Finally the staining was visualized by light microscope (Leica DM-RB and Olympus DP 10 camera).

To exclude any unspecific binding of the secondary antibody, we performed consequently control staining on each transplanted patient renal biopsy sections. The control sections were not incubated with the primary antibodies.

3. Results

3.1. Optimization of AR method

To retrieve the maximal expression of our interest molecules in renal PTECs, it is important to optimize the AR in case of all three molecules (cubilin, megalin and albumin). Therefore, we first optimized the AR method. We have tested 7 AR methods and we tested the IHC staining on biopsy sections without AR pretreatment as well. Then, we performed the IHC staining on 3 different patients renal tissue biopsy specimens and detected the signal with 40x magnification length under light microscopy.

3.1.1. Optimization of megalin expression

To optimize the AR for megalin expression, 3 different patients renal biopsy specimens were subjected to IHC staining. Figure 5 is a representative picture of megalin expression in one patient. Similar staining was observed in the case of the other 2 patients as well (not shown). The primary antibody was diluted in 1:60. We observed that all the AR methods allow a good and suitable staining for megalin. According to the literature, megalin was mainly observed on the apical surface of PTEC with a slight cytoplasmic positivity and the distal tubules were negative (Figure 6) for megalin staining.
Based on our experiments, we ensured citrate D is the best AR method for the detection of megalin by subsequent IHC staining.

3.1.2. **Optimization of cubilin expression**

To optimize the AR method for cubilin expression, 3 different patients renal biopsy specimens were subjected to IHC staining. Figure 7 is a representative picture of cubilin expression in one patient. Similar staining was observed in the case of the other 2 patients as well (not shown). The primary antibody was diluted in 1:100. We observed that in citrate at 80°C, citrate M and no pretreatment AR methods results in good and optimal staining for cubilin. Cubilin was mainly observed on the apical surface of PTEC with cytoplasmic positivity (Figure 8) and the distal tubules were negative for cubilin.
Based on our experiments, we ensured citrate at 80°C is best AR method for the detection of cubilin by subsequent IHC staining.

3.1.3. **Optimization of albumin uptake in PTEC**

To optimize the AR method for albumin uptake, 3 different patients renal biopsy specimens were subjected to IHC staining. Figure 9 is a representative picture of albumin uptake in one patient. Similar staining was observed in the case of the other 2 patients as well (not shown). The primary antibody was diluted in 1:400. Since albumin is an abundant protein, we observed a good and very strong staining for albumin through the sections. In case of no pretreatment and in citrate buffer at 80°C, a strong, brown background was present. Albumin was mainly observed in the proximal tubules (strong, granular cytoplasmic positivity and apical surface staining) (Figure 10). The distal tubules were negative for albumin staining.
Figure 9 illustrates albumin staining on renal biopsy specimens pretreated with different AR methods. The primary antibody dilution was 1:400. Albumin shows apical and cytoplasmic positivity in the PTEC. (Magnification length is 40x)

Based on these experiments, we ensured citrate D is best AR method for the detection of albumin by subsequent IHC staining.

![Citrate D, Citrate at 80°C, Citrate M, Urea](image)

**Figure 9** illustrates albumin staining on renal biopsy specimens pretreated with different AR methods. The primary antibody dilution was 1:400. Albumin shows apical and cytoplasmic positivity in the PTEC. (Magnification length is 40x)

3.2. Optimization of primary antibody dilution

After we optimized the AR method, we investigated the optimal dilutions of the primary antibodies using 3 different patients renal biopsy specimens. We tested different antibody titers for megalin, cubilin and albumin as well.

3.2.1. Optimization of megalin expression

To identify the optimal megalin signal, we tested different primary antibody titers such as 1:30, 1:60 and 1:120. The AR method was citrate D that we determined in the previous experiment. **Figure 10** is a representative picture of megalin staining in one patient. The predicted antibody titer for the detection of megalin was 1:60 because the titer of 1:30 resulted in very strong and quickly developed staining and the titer of 1:120 resulted in a weak signal. Similar staining was observed in the case of the other 2 patients as well (not shown).

![Megalin staining](image)

**Figure 10** illustrates albumin staining on renal biopsy specimen

3.2. Optimization of primary antibody dilution
3.2.2. **Optimization of cubilin expression**

To identify the optimal staining signal of cubilin, we examined the 3 different primary antibody titers such as 1:50, 1:100 and 1:200. The AR method was citrate at 80°C that we optimized previously. **Figure 12** is a representative picture of cubilin staining in one patient. The predicted antibody titer for the detection of cubilin was 1:100. We observed a maximum signal in 1:100 dilution. The titers of 1:50 and 1:200 resulted in weak signals. Similar staining was observed in the case of the other 2 patients as well (not shown).

3.2.3. **Optimization of albumin uptake**

To identify the optimal albumin signal, we tested different primary antibody titers such as 1:200, 1:400 and 1:800. The AR method was urea that we determined in the previous experiment. **Figure 13** is a representative picture of albumin staining in one patient. The predicted antibody titer for the detection of albumin was 1:400 because the titer of 1:200 resulted in strong and quickly developed staining and the titer of 1:800 resulted in a slightly weaker signal. Similar staining was observed in the case of the other 2 patients as well (not shown).
3.3. Expression of megalin and cubilin and uptake of albumin in PTEC of PSI-treated renal transplant patients

Receptor-mediated endocytosis of PTEC is essential in the reabsorption of proteins including albumin. Cubilin and megalin are well-known albumin receptors and play a crucial role in the receptor-mediated endocytosis. We hypothesize that proteinuria occurrence in PSI-treated transplanted patients might be the result of impaired expression of cubilin and/or megalin.

To study the effect of PSI on proximal tubular function, we consequently analyzed and compared the expression level of megalin and cubilin and albumin uptake on kidney biopsy specimens of 4 renal transplanted patients. The biopsy specimens were taken before and during ongoing treatment with PSIs. In case of patient 1, 2 and 3 (as indicated in the text below), no proteinuria was observed during PSI treatment. Patient 4 (as indicated in the text below) suffered from proteinuria after the PSI introduction. Patient 1, 2 and 4 were treated with ERL and patient 3 received SRL. The expression of megalin, cubilin and albumin uptake was investigated by IHC staining.

3.3.1. Analysis of megalin expression

To analyze the impact of PSI medication on megalin expression, we studied serial kidney biopsy specimens of 4 renal transplant patients. Biopsies were taken from all 4 patients before (a) and after introduction of PSI (b). Based on the previous optimization experiments, the biopsies were pretreated with citrate D and the primary antibody was used in the dilution of 1/60. Figure 14 shows the histological analysis of the renal biopsies. In all 4 patients, a continuous expression of megalin on the apical surface and a slight cytoplasmic staining of PTEC were observed. We found in case of patient 1, 2 and 3 that the megalin expression during PSI treatment was comparable or even more intense (b) than before the introduction of PSI (a). However, the expression of megalin was markedly reduced in case of patient 4 during the ongoing PSI treatment. No megalin expression was observed in the distal tubules. Control sections (no primary antibody) did not show immunostaining (picture is not shown).

Figure 14 shows the histological analysis of renal biopsies of 4 renal transplant patients. Figures (a) represent the biopsies taken before the PSI was introduced and figures (b) represent the biopsies taken during ongoing PSI treatment. Specimens were observed under light microscopy and the magnification length was 40x.
3.3.2. Analysis of cubilin expression

To examine the influence of PSI introduction on cubilin expression, we studied serial kidney biopsy specimens of 4 renal transplant patients. From all the 4 patients biopsies were taken before (a) and during medication with a PSI (b). Based on the previous optimization experiments, the biopsies were pretreated with citrate at 80°C and the primary antibody was used in the dilution of 1/100. Figure 15 demonstrates the histological analysis. A continuous apical expression and a strong cytoplasmic staining of cubilin were observed in all 4 patients. We found in case of patient 1, 2 and 3 that the cubilin expression during PSI treatment (b) was comparable to the expression level before the PSI was introduced (a). However, the expression of cubilin during the ongoing PSI medication was considerably decreased in case of patient 4. Similarly to megalin, no cubilin expression was found in the distal tubules. Control sections (no primary antibody was added) did not show immunostaining (picture is not shown).

![Figure 15](image)

3.3.3. Analysis of albumin uptake

From all the 4 patients biopsies were taken before (a) and after ongoing treatment of PSI (b). Based on the previous optimization experiments, the biopsies were pretreated with citrate D and the primary antibody was used in the dilution of 1/400. Figure 16 illustrates the histological analysis of the renal biopsies. Since albumin is a very abundant protein, we observed a high background (brown color) in every biopsy specimens. In all 4 patients a strong staining of albumin was observed on the apical surface and in the cytoplasm of PTEC. The cytoplasmic staining showed a granular appearance. We found in case of patient 1, 2 and 3 that the albumin uptake was comparable or even slightly more intense during PSI treatment (b) than before the introduction of PSI (a). However, the albumin staining was considerably weaker in case of patient 4 during the ongoing PSI treatment. Similarly to megalin and cubilin expression, no albumin uptake was found in the distal tubules. Control sections (no primary antibody was added) were did not show immunostaining (picture is not shown).
Figure 16 shows the histological analysis of biopsies from 4 renal transplant patients. Figures (a) represent the biopsies taken before the PSI was introduced and figures (b) represent the biopsies taken during ongoing PSI treatment. Specimens were observed under light microscopy and the magnification length was 40x.

4. Discussion

In the present study, we investigated the expression of cubilin and megalin and uptake of albumin in the kidney of 4 renal transplanted patients before and after conversion to a PSI. We performed IHC staining on serial kidney biopsies taken before and after introduction of a PSI. We have learned from the medical records that one patient (patient 4) suffered from proteinuria after conversion to a PSI (ERL). In that patient, comparison between the biopsies taken before and after conversion to ERL indicated a markedly reduced cubilin and megalin expression and lack of albumin in the PTECs. The other 3 patients did not suffer from proteinuria and the expression level of cubilin and megalin and the uptake of albumin were comparable on the biopsies taken before and after conversion to a PSI.

In this current study, first we optimized the AR method and primary antibody dilution for three molecules (megalin, cubilin and albumin) present on the FFPE renal tissue sections. During the formalin fixation, formalin may induce cross-linking bridges between the unrelated proteins to target antigens (66). This results in partial or complete loss of immunoreactivity of target antigen referred as masked antigen. The AR methods may allow restoring the immunoreactivity of target antigen. However, the exact mechanism of action still remains largely unknown (66). The optimization of primary antibody titer allows the maximum specific staining with the least amount of background. Specific dilutions may contribute to the quality of staining (67).

In case of albumin staining, we have discussed the problems of the albumin staining with a pathologist of our department and we learned that cryopreservation is the best way to conserve albumin. However, our study is a retrospective study, which means that we could not plan in forward the conservation method, which we would like to use. Therefore, the routinely, in formalin fixed biopsy specimens were available for us. Furthermore, the fluorescent-conjugated antibodies against human albumin provide the best signal. However, on the market there is no such an antibody for the use in formalin fixed and paraffin embedded samples.

Proteinuria can occur after de novo immunosuppression with PSI or after conversion from CNI to PSI (41, 42, 43). Aliabadi et al showed the significant increase in proteinuria in long-term cardiac
transplant patients when immunosuppression was switched from CNI to SRL (41). The fact that proteinuria can be reverted by PSI withdrawal, (43, 49) suggests the direct role of PSI in the development of proteinuria. In our study, we found impaired tubular reabsorption of albumin in a renal transplant patient (patient 4) who has developed proteinuria during ongoing treatment with PSIs. We showed down regulated expression of megalin and cubilin and reduced albumin uptake in the same proteinuric renal transplant patient during immunosuppression treatment with a PSI. We can distinguish two types of proteinuria; one is glomerular proteinuria, which is the result of increased glomerular permeability and the other is tubular proteinuria. In vitro studies of Beate vollenbroker et al suggests that rapamycin may induce the podocyte damage by down regulating the expression of slit-diaphragm proteins (nephrin and TRPC6). Rapamycin affects the mTOR signaling pathway, which results induction of podocyte damage (50) leads to proteinuria. The tubular proteinuria can be the result of impaired reabsorption of proteins or tubular toxicity. However, our study aimed to investigate the role of tubular proteinuria and the underlying mechanism in PSI treated transplant patients.

The reduced expression of megalin and cubilin in the proximal tubules strongly indicates that the receptor-mediated albumin uptake is impaired during PSI treatment and might lead to proteinuria. A case study supports our observation. Straathof-Galema et al reported proteinuria and complete lack of albumin in PTEC in a renal transplant patient during immunosuppression therapy with a PSI (49). Furthermore it is well known that both, megalin and cubilin are involved in the reabsorption of albumin in the kidney (2, 31). Megalin deficient mice showed increased excretion of proteins (32). Severe proteinuria was observed in patients with hereditary malabsorption of vitamin B12, which is a result of structurally abnormal expressed cubilin (35).

In patient 4, during ongoing treatment with ERL cytomegalovirus (CMV) infection was observed (Table 1). CMV infection may be also associated with proteinuria. In fact, CMV infection is common after transplantation and associated with cardiovascular diseases. One study indicated that CMV infection might cause increased arterial blood pressure (61). Valantine H suggests CMV infection may able cause initiation of allograft injury leads to cardiac allograft vasculopathy. CMV infects the cells (smooth muscle and endothelial cells, moncytes, macrophages), which involved in the development of atherosclerosis (70). Liciano. P et al demonstrated that, CMV prophylaxis reduces the incidence of acute rejection and cardiac allograft vasculopathy disease (69). In renal transplantation gender mismatch between the donor and recipient may be also associated with increased risk of proteinuria after PSI therapy. Adrian. L et al showed that increased risk of proteinuria when the male recipients received an allograft from female donor (53). However in our study, patient 4 (female) received an allograft from male donor.

We analyzed the expression of megalin, cubilin and albumin uptake in the other three renal transplanted patients (patient 1, patient 2 and patient 3) as well. As mentioned earlier, we found in patient 1, 2 and 3 that the megalin expression during PSI treatment was comparable or even more intense than before the introduction of PSI. We did not find significant difference in the expression level of cubilin between before and during ongoing treatment with PSI. Patient 2 and patient 3 got angiotensin converting enzyme inhibitors (ACE-inh) (enalapril) (Table 6). Whether patient 1 got ACE-inh or ARB, it is not known. According to patient’s medical records, in patient 1 and 3 proteinuria was not observed before and during PSI treatment. Patient 2 suffered from proteinuria already before the PSI was introduced and proteinuria was restricted during the treatment with PSI.
As earlier mentioned, patient 2 got ACE-inh during ongoing PSI treatment. We suspect that ACE-inh might have an effect on the reduction of proteinuria in patient 2 and in the improved or unchanged megalin expression. ACE-inh blocks the conversion of Ang I to Ang II. Ang II is known to cause hypertension. ACE-inh lowers the blood pressure and has a favorable effect in proteinuria treatment. A large number of retrospective studies found the ACE-inh or angiotensin receptor blockers show considerable effects in the reduction of urinary protein loss in transplanted patients during PSI therapy and in diabetic or non-diabetic nephropathy patients as well (59, 63). ACE-inh protects the kidney by reducing the glomerular hypertension and blocking other Ang II-mediated effects. In our previous study, we found that ACE-inh (ramipril) or ARB (losartan) prevents the loss of cubilin and megalin and improve albumin uptake in the presence of PSIs on cultured PTEC (Oroszlan M et al). In addition, other studies suggest that Ang II plays an important role in the regulation of megalin expression (36, 63).

The proteinuric patient 4 got ACE-inh as well. Despite of that medication, proteinuria developed during PSI treatment. The proteinuria occurrence may affected by demographic parameters, preexisting diseases, body mass index (BMI) and variations of the medications. SRL, ERL have a target blood level 10-12 ng/ml and 6-8 ng/ml, respectively. In reality however, the trough level or tissue concentration of these drugs can be several fold higher and might have a serious clinical consequences (65). Unfortunately, the trough levels of the drugs in our patients are not known.

In conclusion, we have found that a renal transplant patient developed proteinuria during medication with ERL. The histological analysis of the patient 4 biopsies showed a markedly reduced cubilin and megalin expression and reduced albumin uptake in PTEC. We speculate that there is a direct link between PSI medication and tubular dysfunction, which might cause proteinuria.

Further studies are needed to understand how cubilin and megalin expression is regulated by PSI. We don’t know yet whether the decreased expression is due the protein degradation or decreased gene expression. Furthermore, the exact role of Ang II has to be cleared as well.

Limitation of the study

The number of patients involved in the study is low. To make a firm conclusion, the study requires more patients.

5. References


50. Beate Vollenbroeker, Britta George, Maria Wolfgart, Moin A. Saleem, Hermann Pavenstaedt1 and Thomas Weide, (2008): mTOR regulates expression of slit diaphragm proteins and


52. Stracke S, Mayer JM, Henne-Bruns D, Keller F (2006): Elevated serum levels of vascular endothelial growth factor (VEGF) and proteinuria in renal allograft recipients treated with mTOR inhibitors. Transplantation 2006;82(suppl 2):299.


