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PROTEIN UPTAKE AT GLOMERULAR LEVEL: POSSIBLE INVOLVEMENT OF AN ENDOCYTIC MACHINERY IN CELL CULTURE AND IN PATIENTS WITH LUPUS NEPHRITIS

Coordinatore: Ch.mo Prof. Gaetano Thiene
Supervisore: Dott.ssa Dorella Del Prete

Dottorando: Dott.ssa Lisa Gianesello

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1. Abstract

1.1. ABSTRACT

ClC-5 with megalin (LRP2), cubilin, Disabled 2 (Dab2), and Amnionless (AMN), is part of the molecular complex involved at proximal tubular level in the endocytic re-uptake of low-molecular-weight proteins and albumin. ClC-5, megalin and cubilin expression in podocytes of human renal biopsies was already reported. Moreover, it was demonstrated that podocytes are able to internalize albumin through an endocytic process. It is reasonable to assume that there should be an involvement of this system in protein uptake mediated by podocytes. A disruption of this system can lead to proteinuria which is one of the first manifestation of kidney disease in Systemic Lupus Erythematous (SLE).

Aims of this study were to explore the presence of the tubular endocytic machinery components also in human podocytes *in vitro* and to evaluate whether albumin modulates this system. Moreover, we wanted to verify and quantify the expression of ClC-5, megalin and cubilin in both glomerular and tubular compartments in renal biopsies of patients presenting Lupus nephritis and observing the presence of a relationship with clinical data.

To verify the presence of an uptake mechanism in human podocytes *in vitro*, we performed time lapse experiments with a low dose of FITC albumin (10 μg/ml). We observed albumin internalization starting from 2 to 15 hours. To evaluate the uptake kinetic, we stimulated podocytes at different time (30 min and 2 hours) and doses (10 μg/ml, 100 μg/ml and 1 mg/ml) at 37°C and 4°C. We observed a significant dose dependent increase in fluorescence vs controls after 2 hours' stimulation with a typical receptor-mediated kinetic since it is inhibited at 4 °C. Moreover, we disclosed the presence of ClC-5, Dab2 and AMN beyond megalin and cubilin, in this *in vitro* system using immunohistochemistry (IHC) and immunofluorescence (IF) techniques, highlighting a co-localization between albumin and both receptors. To analyze whether the proteinuric environment modulates *CLCN5*, *LRP2*, *CUBN*, *DAB2* and *AMN* expression, we stimulated human podocytes with increasing concentrations of BSA (range 10 μg/ml - 30 mg/ml) and we evaluated the mRNA expression at different time points (2, 4, 8, 24, 48 and 72 hours). Using Real Time PCR we observed a significant time and dose dependent increase in *CLCN5*, *CUBN* and *AMN* expression and an up-regulation of DAB2 only at 24 hours.

We collected 23 SLE renal biopsies, 6 control biopsies and 1 case of Minimal Change Disease. As clinical parameters we considered proteinuria and pharmacological therapy. IHC and IF were used to analyze ClC-5, megalin and cubilin protein expression in serial sections. Morphometric quantification revealed a direct correlation between tubular and glomerular expression of all molecules in SLE patients, highlighting a relationship between glomerular and tubular compartments independently from proteinuria levels. Furthermore, preliminary data on patients

without ACEi/ARB and immunosuppressive drugs disclosed a positive trend among these molecules at glomerular level.

Interestingly, we revealed megalin and cubilin expression in hypertrophic PECs of some SLE patients and characterization experiments identified a subpopulation with an intermediate phenotype between mature and progenitor cells.

In conclusion, for the first time we demonstrated that human podocytes are naturally committed to perform albumin endocytosis via a receptor-mediated mechanism. Moreover, protein overload upregulates *CUBN*, *AMN* and *CLCN5* in these cells.

Functional studies regarding the role of cubilin in albumin uptake underlined its participation in this mechanism even if this is not the only pathway involved. Further studies will be necessary to analyze which may be the partner(s) of the CUBAM complex in this mechanism.

For the first time we demonstrated the presence of ClC-5, megalin and cubilin in glomeruli of patients with SLE and MCD in addition to controls confirming *in vitro* data. Furthermore, in SLE biopsies we highlighted a strong correlation between the two renal compartments in the expression of the protein uptake system, supporting the idea of a partnership between tubular and glomerular cells in albumin uptake *via* the same mechanism of internalization. In addition, preliminary data on patients without ACEi/ARB and immunosuppressive drugs lead us to suppose that the pharmacological therapy could affect the expression of this system, in particular at glomerular level.

The differences observed from *in vivo* and *in vitro* data, especially on megalin expression, underline the involvement of other glomerular cell types in addition to podocytes in protein uptake.

Finally, megalin and cubilin expression in PECs of SLE patients is a real interesting but complex data, since characterization experiments identified a subpopulation with an intermediate phenotype between mature and progenitor cells. Further studies will be performed to better characterize the role of these double-positive cells and their correlation with clinical data and/or disease progression.

1.2. RIASSUNTO

ClC-5, megalina (LRP2), cubilina, Disabled 2 (Dab2) ed Amnionless (AMN) fanno parte del complesso molecolare coinvolto a livello del tubulo prossimale nel recupero delle proteine a basso peso molecolare e dell'albumina mediante endocitosi. È già stata riportata la presenza di ClC-5, megalina e cubilina a livello dei podociti in biopsie renali umane. Inoltre è stato dimostrato che i podociti sono in grado di internalizzare l'albumina attraverso un meccanismo di endocitosi. Il mancato funzionamento di questo sistema può portare a proteinuria, che è una delle prime manifestazioni del coinvolgimento renale nel Lupus Eritematoso Sistemico (LES). È quindi ragionevole supporre che vi possa essere un coinvolgimento di questo sistema nel meccanismo di uptake delle proteine da parte dei podociti.

Gli scopi di questo studio sono stati di esplorare la presenza dei componenti del sistema tubulare di endocitosi delle proteine in podociti umani in coltura e di valutare se e come l'albumina ne modulasse l'espressione. Inoltre, si è voluto indagare l'espressione di ClC-5, megalina e cubilina sia a livello glomerulare che tubulare in biopsie renali di pazienti con nefrite lupica, valutando una possibile relazione con i dati clinici.

Abbiamo verificato la presenza di un meccanismo di uptake in podociti umani in coltura attraverso esperimenti di time-lapse con basse dosi di FITC-BSA (10 μg/ml) ed abbiamo osservato l'inizio del processo di internalizzazione in un periodo di tempo variabile dalle 2 alle 15 ore. Per caratterizzare il tipo di cinetica di uptake della FITC-BSA, i podociti sono stati stimolati a differenti tempi (30 min e 2 ore) e dosi (10 μg/ml, 100 μg/ml and 1 mg/ml) mantenendo la coltura a 37°C o 4°C. Si è osservato un aumento significativo della fluorescenza dose-dipendente rispetto al controllo dopo 2 ore dalla stimolazione con una tipica cinetica di internalizzazione recettore-mediata poiché veniva inibita a 4°C. Abbiamo osservato la presenza di ClC-5, Dab2 e AMN oltre a quella di megalina e cubilina in podociti umani in coltura in condizioni basali mediante tecniche di immunoistochimica (IHC) ed immunofluorescenza (IF) ed abbiamo dimostrato la co-localizzazione dei due recettori con l'albumina fluorescente. Per valutare se l'ambiente proteinurico fosse in grado di modulare l'espressione di *CLCN5*, *LRP2*, *CUBN*, *DAB2* ed *AMN*, i podociti umani sono stati stimolati con concentrazioni crescenti di BSA (range 10 μg/ml - 30 mg/ml) e l'espressione dell'RNA messaggero è stata valutata a tempi diversi (2, 4, 8, 24, 48 and 72 hours). Mediante analisi in Real Time PCR, abbiamo osservato un aumento significativo tempo e dose-dipendente di *CLCN5*, *CUBN* ed *AMN* ed un aumento di *DAB2* solamente alle 24 ore.

Abbiamo raccolto 23 biopsie renali di pazienti con LES, 6 biopsie di controllo ed un caso di Minimal Change Disease. Come parametri clinici abbiamo considerato la proteinuria e la terapia farmacologica. Mediante IHC ed IF abbiamo analizzato

l'espressione proteica di ClC-5, megalina e cubilina in sezioni seriali. La quantificazione eseguita mediante analisi morfometrica ha rivelato una correlazione diretta dell'espressione di tutte le molecole in analisi tra il compartimento tubulare e glomerulare, evidenziando una stretta relazione tra i due compartimenti indipendentemente dai livelli di proteinuria. Inoltre, dati preliminari su pazienti privi di terapia farmacologica (ACEi/ARB o immunosoppressivi) hanno mostrato un trend positivo tra l'espressione di queste molecole a livello glomerulare.

Curiosamente, abbiamo evidenziato l'espressione di megalina e cubilina in cellule parietali della capsula (PECs) con morfologia ipertrofica in alcuni pazienti LES che, mediante esperimenti di caratterizzazione, abbiamo identificato come una nuova sottopopolazione con un fenotipo intermedio tra cellule mature e progenitrici.

Concludendo, per la prima volta abbiamo dimostrato che i podociti umani sono naturalmente predisposti ad effettuare l'endocitosi dell'albumina attraverso un meccanismo recettore-mediato. Inoltre, l'overload proteico è in grado di aumentare l'espressione di *CLCN5*, *CUBN* ed *AMN* in queste cellule.

Studi funzionali per dimostrare il ruolo di cubilina nel processo di uptake dell'albumina hanno sottolineato la sua partecipazione in questo meccanismo anche se, verosimilmente, non è l'unico pathway coinvolto. Ulteriori studi saranno necessari per analizzare quali altre molecole possano essere chiamate in causa in questo meccanismo.

Per la prima volta abbiamo dimostrato la presenza di ClC-5, megalina e cubilina in glomeruli di pazienti con LES, MCD e controlli, confermando i dati *in vitro*. Inoltre, nelle biopsie dei pazienti LES abbiamo evidenziato una stretta relazione tra i due compartimenti renali nell'espressione dei componenti di questo sistema, supportando l'idea di una partnership tra cellule tubulari e glomerulari nell'uptake dell'albumina attraverso lo stesso meccanismo di internalizzazione. In aggiunta, dati preliminari ottenuti da pazienti privi di terapia con ACEi/ARB o immunosoppressivi ci ha fatto supporre che il trattamento farmacologico possa influire sull'espressione di questo sistema a livello glomerulare. Le differenze osservate tra lo studio *in vivo* e quello *in vitro*, in particolare riguardo l'espressione di megalina, suggeriscono il coinvolgimento di altre cellule del glomerulo oltre ai podociti.

Infine, l'espressione di megalina e cubilina nelle PECs dei pazienti LES è un dato molto interessante ma complesso, poiché gli esperimenti di caratterizzazione hanno identificato una sottopopolazione con un fenotipo intermedio tra cellule mature e progenitrici. Ulteriori studi dovranno essere condotti per meglio caratterizzare il ruolo di queste cellule con doppia positività e la loro correlazione con i dati clinici o di progressione della malattia.

2. Background

2.1. THE NEPHRON

The nephron is the basic structural and functional unit of the kidney and comprises the renal corpuscle (Bowman's capsule and glomerulus) and the renal tubule. The proximal renal tubule is the first portion of the nephron to modify ultrafiltrate. It accounts normally for all resorption of proteins, amino acids, glucose and creatinine and most resorption of water, as well as Cl^- and Na^+ ions.

Proximal tubular epithelial cells (PTECs) are tall and have a dense brush border of microvilli that greatly expand the area available for absorption. At the bases of the microvilli, the cell membrane is pitted extensively and displays numerous apical canaliculi that lead into the cytoplasm. This network of pits and tubules is underlined by vesicles representing various stages of endocytosis and is related to resorption of proteins from the ultrafiltrate (Maunsbach, 1976).

At first glance the glomerulus is a simple filtering structure connected to a couple of pipes but on closer inspection, advancement in science has revealed much of the beauty and complexity of this structure. Each individual glomerulus works with its millions of counterparts adjusting to a multitude of physiologic disruptions, reliably helping to maintain homeostatic equilibrium. Yet the glomerulus is also prone to injury, particularly related to autoimmunity and inflammation (Jhaveri and Fishabane, 2014).

At glomerular level we can find several cell types: parietal epithelial cells, podocytes, endothelial cells and mesangial cells (Figure 1). Mesangial cells are surrounded by an extracellular matrix (mesangial matrix) that lies between the mesangial and endothelial cells or the glomerular basement membrane (GBM) (Vize et al., 2003).

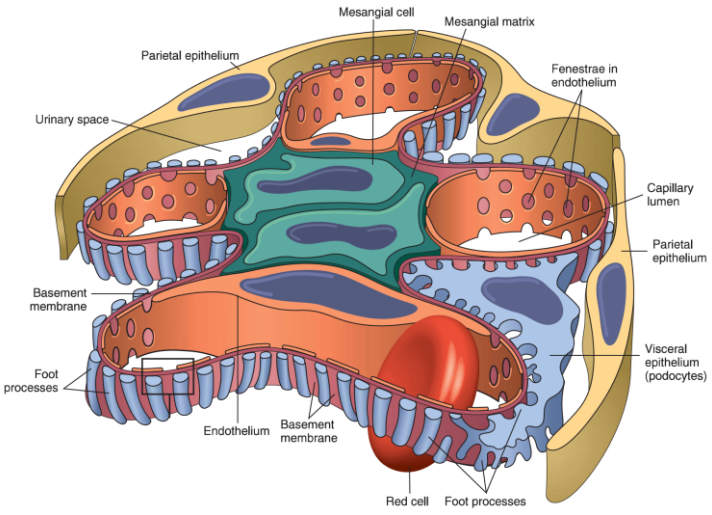


Figure 1. Glomerular cells. *Diagrammatic representation of the different cells composing glomeruli (modified from Kumar et al., 2013).*

2.1.1. The glomerular filtration barrier

In humans, close to 180 liters of primary urine are produced each day at capillary pressures far exceeding those in other organs. Despite this tremendous work load, the glomerulus remains intact year after year (Haraldsson et al., 2008). Glomerular filtration is the initial step of urine formation. The capillary wall differs from other capillaries by having three instead of two layers (Figure 2).

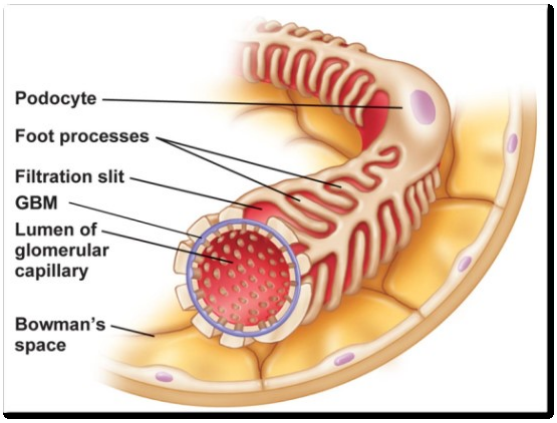


Figure 2. Glomerular filtration barrier. *Diagrammatic representation of the components of the glomerular filtration barrier. Copyright © Pearson Education Inc. 2011 (modified).*

A fenestrated endothelium, similar to that found in the liver, partially covers the GBM, and its importance is generally underappreciated (Jhaveri and Fishabane, 2014). These fenestrations allow the endothelial cells to serve as a barrier against cellular components of blood and large macromolecules (Vize et al., 2003).

The glomerular filtration rate (GFR) is proportional to the total area of fenestrations, and disorders of fenestrations play a role in proteinuria and loss of renal function. The second layer of the capillary wall, the GBM, is composed of a variety of proteins, most importantly type IV collagen, which forms the membrane's ultrastructure. The GBM serves several functions, including vital aspects of size and charge filtration selectivity. The third and final component of the capillary wall is the epithelial cell or podocyte, a terminally differentiated cell that projects foot processes over the GBM. Gaps, or slits, between these cells, covered by thin-slit diaphragms, are the route for regulated filtration. The podocyte has a critical role in the filtration barrier, forming a highly organized structure, disorders of which cause proteinuria (Jhaveri and Fishabane, 2014).

The glomerular barrier is by far the most complex biological membrane, with properties that allow for high filtration rates of water, non-restricted passage of small and middle-sized molecules, and almost total restriction of serum albumin and larger proteins (Haraldsson et al., 2008). This elegant structure has to oppose hydrostatic pressure in the glomerular capillary, which is the natural driving force behind macromolecular filtration (Reiser and Altinas, 2016). Taken together the glomerular

capillary wall is a remarkable structure that allows for high-volume filtration while maintaining charge and size selectivity and preventing loss of significant quantities of protein (Jhaveri and Fishabane, 2014).

2.1.2. Podocytes

2.1.2.1. Podocytes biology

Podocytes (or visceral epithelial cells) are highly differentiated cells lining the outer surface of the glomerular capillaries. They have a voluminous cell body, which bulges into the urinary space (Pavenstädt et al., 2003).

As a major component of the ultrafiltration apparatus, podocytes have a complex cellular architecture consisting of cell body and major processes that extend outward from their cell body, forming interdigitated foot processes that enwrap the glomerular capillaries. Major processes are tethered by microtubules and intermediate filaments while foot processes contain actin-based cytoskeleton (Reiser and Altinas, 2016). The cell body contains a prominent nucleus, a well-developed Golgi system, abundant rough and smooth endoplasmic reticulum, prominent lysosomes, and many mitochondria. In contrast to the cell body, the cell processes contain only a few organelles. The density of organelles in the cell body indicates a high level of anabolic as well as catabolic activity. Moreover, a well-developed cytoskeleton accounts for the unique shape of the cells and the maintenance of the processes (Pavenstädt et al., 2003).

Podocyte function depends on a highly ordered cellular arrangement of filtration compartments and the correct signaling within this microenvironment. Therefore, podocytes are uniquely sensitive to a variety of agents interfering with their actin cytoskeleton, their apical membrane domain (i.e., the negative surface charge), slit diaphragm complex that regulates podocyte actin reorganization, and GBM structure to which podocytes adhere. (Reiser and Altinas, 2016).

2.1.2.2. The slit diaphragm

Podocytes are polarized epithelial cells with a luminal (or apical) and a basal cell membrane domain. They form a tight network of interdigitating cellular extensions, called foot processes, which are bridged by so-called “slit diaphragms” (Pavenstädt et al., 2003) (Figure 3). The core structural component of podocyte foot processes is a highly regulated actin cytoskeletal network, which was represented either by a dense bundle of actin filaments that extends along the length of foot processes or by

a relatively short and branched cortical network, which is located at the cell periphery and anchors elements of the slit diaphragm (Reiser and Altinas, 2016). The luminal membrane and the slit diaphragm are covered by a thick surface coat that is rich in sialoglycoproteins, including podocalyxin and others, which are responsible for the high negative surface charge of the podocytes (Sawada et al., 1986). Despite being of very delicate appearance, properly functioning slit diaphragms seem to be a crucial prerequisite to produce virtually protein-free urine (Grahammer et al., 2013).

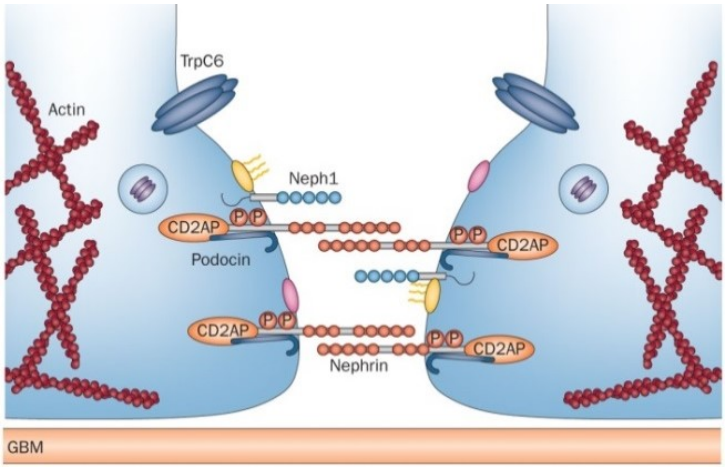


Figure 3: The slit diaphraghm. *Diagrammatic representation displaying proteins involved in the slit diaphragm (Modified from Grahammer et al., 2013).*

The filtration barrier is freely permeable by water and small solutes, but to a large extent, the size selectivity of the filtration barrier for proteins is represented by the slit diaphragms of podocytes (Pavenstädt et al., 2003). In the past few years, much has been learned about the molecular architecture of the slit diaphragm. During development, podocytes are initially connected via tight-junction and gap-junction components. Gradually, these components are replaced by the neuronal-junction components, nephrin, podocin and Neph1. These proteins form a zipper-like structure that is the hallmark of the mature slit diaphragm (Grahammer et al., 2013). Nephrin is the major component of the slit diaphragms between adjacent foot processes binding to each other through disulfide bridges at the center of the slit diaphragm. The intracellular part of nephrin interacts with several cytoskeletal and signaling proteins (Figure 3). Nephrin and its associated proteins, including podocin, have an important role in maintaining the selective permeability of the glomerular filtration barrier. Its importance is underlined by rare hereditary diseases in which mutations of nephrin or its partner proteins are associated with abnormal leakage into the urine of plasma proteins, leading to the nephrotic syndrome. This observation suggests that defects in the function or structure of slit diaphragms constitute an important mechanism of proteinuria (Kumar et al., 2013, Gianesello et al., in press).

In addition, foot processes have a thick, negatively charged coat (glycocalyx) facing the urinary space; this accounts for negative surface charges throughout the glomerular filtration barrier, which generates an electrostatic repel between the neighboring foot processes and helps maintain the unique cytoarchitecture of podocytes by enhancing the physical separation (Reiser and Altinas, 2016).

Protein components of the slit diaphragm have to fulfil at least four different tasks: they act as a macromolecular filter; anchor the filter to the GBM; connect the slit diaphragm, *via* adaptor proteins, to the actin cytoskeleton; and are part of a signaling complex that integrates and mediates extracellular and intracellular signals regulating the plasticity of foot processes. To facilitate all the aforementioned tasks, the slit diaphragm integrates structural stability, foot process guidance and the control of cell polarity. Thus, the slit diaphragm can not only be regarded as a static sieve linking adjacent foot processes, but also as representing a dynamic signaling hub that transfers and integrates diverse intracellular and extracellular signals (Grahammer et al., 2013).

2.1.2.3. The foot process effacement

Although the podocyte injury is not the only cause of major glomerular diseases, a stable podocyte architecture with interdigitating foot processes connected by highly specialized filtration slits is essential for the maintenance and proper function of the glomerular filtration barrier. If podocytes are injured, mutated, or lost, the elaborate structure of podocytes is physically altered (a process termed 'foot process effacement') which is found in many proteinuric kidney diseases (Reiser and Altinas, 2016).

The initial response of podocytes to stress or injury is the disruption of the foot processes and actin dysregulation, where actin and actin-binding proteins accumulate. In some cases, once foot processes are effaced (flattened down and fused), the glomerular filtration barrier is no longer intact as evidently indicated by the massive leak of proteins out of the vasculature into the urine, known as proteinuria. The mechanisms leading to podocytopathies at the molecular level include genetic events (genetic mutations and deletions) associated with common complex diseases (Grahammer et al., 2013; Reiser and Altinas, 2016; Gianesello et al., in press).

2.1.3. Parietal Epithelial Cells (PECs)

Parietal Epithelial Cells (PECs) are positioned between the main glomerular filtration barrier and the proximal tubule and they are considered to be in a prime position to potentially act as a sensor for kidney health (Chang et al., 2012). Studies using both ultrastructural and immunostaining techniques suggested that, following completion of kidney development, different 'subpopulations' of PECs exist in both healthy and diseased adult kidneys (reviewed in Shankland et al., 2014). To date, a uniform nomenclature for the various cell types lining Bowman's capsule has not been achieved but the literature describes several PEC subpopulations based on either their description (descriptive terminology), on their potential functional role as a kidney progenitor cell (progenitor terminology) or both (Shankland et al., 2014).

In healthy individuals, descriptive terminology (Figure 4, left) define the Bowman's capsule as lined by PECs while, at the vascular pole, PECs are in direct continuity with visceral podocytes; cells with an intermediate phenotype (peripolar cells or transitional cells) can be found between the two (Appel et al., 2009). Cells expressing podocyte markers, with a differentiated podocyte phenotype or both on the Bowman's capsule are called parietal podocytes, which are usually located close the vascular pole. PECs showing larger cytoplasm and nucleus and increased migration and proliferation in different disease states are termed activated PECs (aPECs) (Smeets et al., 2009). Occasionally, vacuolization of aPECs can be observed, possibly representing protein resorption droplets (Ohse et al., 2009a; Smeets and Moeller, 2012).

Mature PECs can be identified by the expression of a set of specific markers such as annexin A3 (ANXA3), Pax-8, Claudin-1, -2 and -16, UCH-L1 and the matrix protein LKIV69 (Kietzmann et al., 2015; Kuppe et al., 2015). Using progenitor terminology (Figure 4, right) and assuming that PECs function as progenitors for podocytes and tubular cells, cells on the Bowman's capsule that express progenitor markers such as CD24 and glycosylated CD133 and no podocyte or tubular cell markers are termed adult parietal epithelial multipotent progenitor (APEMP) (Ronconi et al., 2009; Lasagni and Romagnani, 2013). This subset of multipotent progenitors in the Bowman's capsule may provide an intriguing explanation for the genesis of crescents, which are known to reflect uncontrolled proliferation of PEC and their transdifferentiation into mesenchymal and myeloid cells during rapidly progressive glomerulonephritis (Sagrinati et al., 2006). Moreover, same authors suggested that crescent formation might reflect a dysregulated activation of APEMP in response to chronic inflammatory stimulation (Sagrinati et al., 2006). Cells co-expressing podocyte or tubule markers are designated committed progenitors. Activated PECs can be recognized by the *de novo* expression of CD44 as found in experimental models of FSGS and crescentic glomerulonephritis and in biopsies of human patients

with FSGS and crescentic glomerulonephritis (Smeets et al., 2014). CD44 is the main receptor for osteopontin and hyaluronic acid and is involved in cell adhesion, matrix interaction, and migration. CD44 is also expressed by a subset of mononuclear cells (that is, macrophages and monocytes) but not by resident glomerular cell types (Smeets et al., 2011), delineating this molecule as the only known specific marker identifying aPECs from other parietal cell types in experimental and clinical diseases (Smeets et al., 2009; Smeets et al., 2011; Fatima et al., 2012).

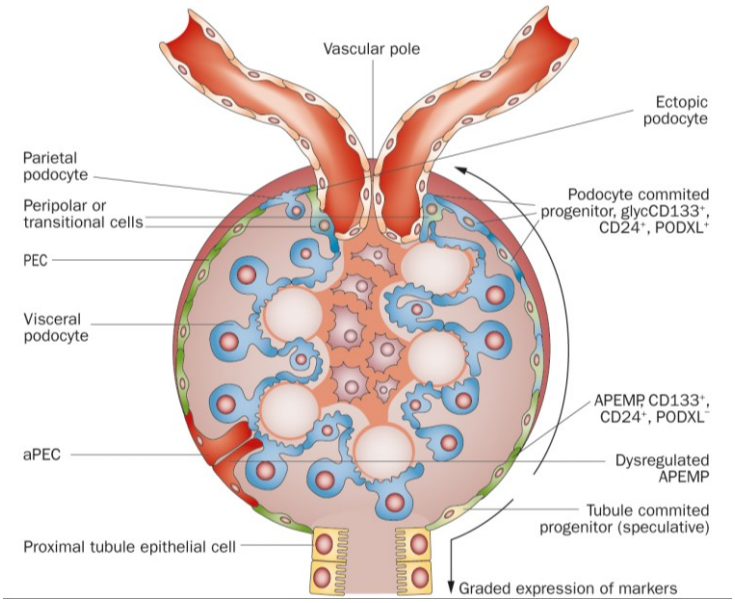


Figure 4. PEC subpopulations. *Classification of PECs populations using descriptive and progenitor terminologies. PODXL: Podocalyxin (Modified from Shankland et al., 2014).*

2.2. ENDOCYTOSIS IN THE KIDNEY

Endocytosis is a highly coordinated process that plays key roles in cell signaling and homeostasis internalizing and retrieving plasma membrane components and transmembrane receptors by forming vesicles (De et al., 2014; Inoue and Ishibe, 2015). Endocytosis can be divided into clathrin-dependent and-independent pathways. Interestingly, proteins can often exploit either pathway to allow for internalization (Inoue and Ishibe, 2015). The most extensively studied and best characterized at molecular level is clathrin-mediated endocytosis, which involves the internalization of cell-surface receptors and soluble molecules, including nutrients, from the extracellular fluid in clathrin-coated vesicles that bud off from the plasma membrane (Scita and Di Fiore, 2010; Taylor et al., 2011).

Although clathrin is the main coat component of the endocytic vesicles, a number of adaptor proteins are also involved in the initiation of vesicle budding, as clathrin is unable to interact directly with lipids and proteins in the plasma membrane. These adaptor proteins are required to link clathrin with the membrane and are also able

to specifically bind the endocytic cargo and ensure its uptake into clathrin-coated vesicles. After internalization, membrane receptors reach early endosome from where they are recycled back to the plasma membrane or go to the late endosomes from where they are destined to lysosomal degradation (De et al., 2014).

2.2.1. Endocytosis in PTECs

In the normal kidney, reabsorption of filtered proteins occurs almost exclusively by receptor-mediated endocytosis in the proximal tubule. The apical endocytic apparatus is very elaborate in the first two segments of the proximal tubule, consisting of coated pits, coated vesicles, endosomes, lysosomes, and dense apical tubules responsible for membrane and receptor recycling from endosomes to the apical plasma membrane (Christensen et al., 2012).

The receptor-mediated endocytosis and subsequent metabolization of proteins and nutrients is considered to be one of the most important functions of renal proximal tubular epithelial cells (PTECs). Receptor-mediated endocytosis requires the coordinated functioning of numerous proteins and signal transduction molecules. In particular, megalin and cubilin play a central role in the process (Kerjaschki and Farquhar, 1982; Sahali et al., 1988). These receptors bind a variety of filtered ligands with varying affinities (Table 1) fulfilling their task by receptor-mediated endocytosis mediating delivery of their ligands to the lysosomes of the proximal tubule, while they undergo recycling themselves (Christensen et al., 1982; Christensen et al., 2009).

Due to the great abundance and efficient recycling of receptors at the apical plasma membrane, the reabsorption is very efficient. In addition to the simple removal of urinary proteins providing a nontoxic, protein-free *milieu* in more distal parts of the nephron and the collecting ducts, this process serves a number of other important physiological functions (Christensen et al., 2012).

The apical plasma membranes of PTECs form a brush border surface that facilitates endocytosis of macromolecules from the lumen of the tubules via clathrin-coated pits. The reabsorption recovers essential substances that otherwise would be lost in the urine, notably vitamins and different trace elements bound to their carrier proteins (Nykjær et al., 1999; Nykjær et al., 2001).

Megalin	References	Cubilin	References
	Vitamin carrier proteins		
Transcobalamin-vitamin B12	115	Intrinsic-factor vitamin B12	158
Vitamin D-binding protein	128	Vitamin D binding protein	129
Retinol-binding protein	40		
Folate-binding protein	19		
	Other carrier proteins		
Albumin	49	Albumin	14
Myoglobin	60	Myoglobin	60
Hemoglobin	61	Hemoglobin	61
Lactoferrin	183	Transferrin	101
Selenoprotein P	133		
Metallothionein	95		
Neutrophil-gelatinase-associated lipocalin	83		
Odorant binding protein	106		
Transthyretin	161		
Liver type fatty acid binding protein	137		
Sex hormone binding globulin	72		
	Lipoproteins		
Apolipoprotein B	163	Apolipoprotein AI	100
Apolipoprotein E	183	High density lipoprotein	100
Apolipoprotein J/clusterin	99		
Apolipoprotein H	119		
Apolipoprotein M	56		
	Hormones and signaling proteins		
Parathyroid hormone	76		
Insulin	134		
Epidermal growth factor	134		
Prolactin	134		
Thyroglobulin	190		
Sonic hedgehog protein	114		
Angiotensin II	64		
Leptin	70		
Bone morphogenic protein 4	162		
Connective tissue growth factor	62		
Insulin like growth factor	21		
	Enzymes and enzyme inhibitors		
Plasminogen activator inhibitor-type I	164		
Plasminogen activator inhibitor-type I-urokinase	118		
Plasminogen activator inhibitor-type I-tissue plasminogen activator	118		
Pro-urokinase	164		
Lipoprotein lipase	98		
Plasminogen	86		
α- amylase	17		
Lysozyme	134		
Cathepsin b	126		
α-galactosidase A	46		
Cystatin C	89		
	Immune- and stress-related proteins		
Immunoglobulin light chains	15	Immunoglobulin light chains	10
Pancreatis associated protein 1	106	Clara cell secretory protein	29
α1-microglobulin	106	α1-microglobulin	165
β2-microglobulin	134		
	Drugs and toxins		
Aminoglycosides	116	Aminoglycosides	169
Polymyxin B	116		
Aprotinin	116		
Trichosantin	32		
	Receptors		
Cubilin	117	Megalin	117
Transcobalamin II-B12 receptor	186		
	Others		
Receptor associated protein	39	Receptor associated protein	16
Ca2+	39		
Cytochrome C	134		
Seminal vesicle secretory protein II	145		
Recombinant activated factor VIIa	157	Recombinant activated factor VIIa	157
Coagulation factor VIII	5		

Table 1. Ligand for megalin and cubilin. *(Modified from Christensen et al., 2012).*

Although proteins are degraded in lysosomes, vitamins and trace elements are returned to the circulation by transport across the basolateral membrane. In addition, reabsorbed substances may be metabolized within PTECs as illustrated by

the important, renal activation of endocytosed 25-OH-vitamin D3 (Nykjær et al., 1999) (Figure 5).

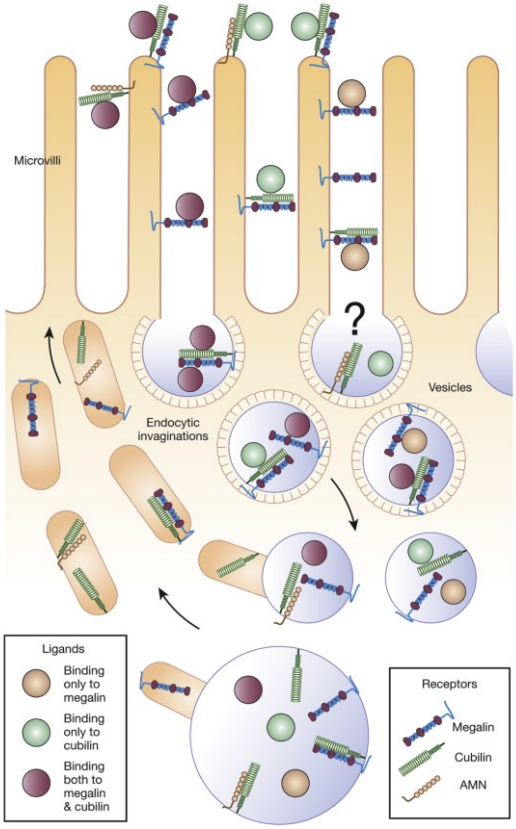


Figure 5. Receptor-mediated endocytosis at tubular level. *Ligands (green, yellow, and purple circles) bind to either megalin or cubilin, or both, with varying affinity (Modified from Nielsen et al., 2016).*

2.2.2. Endocytosis in podocytes

The first evidence of an endocytic process at glomerular level was described in 1986 by Yoshikawa et al. that reported the presence of vacuolation of the epithelial glomerular cells in children with nephrotic syndrome and focal segmental glomerulosclerosis (Yoshikawa et al., 1986). Subsequently, Eyre et al. described and quantified an albumin endocytic function in podocytes both *in vitro* and *in vivo* (Eyre et al., 2007) (Figure 6), and Koop et al. identified protein droplets in podocyte cell bodies and major processes in Dahl salt-sensitive rats (Koop et al., 2008). In puromycin aminonucleoside nephrotic rats, it was also demonstrated that albumin is filtered *via* other pathways beyond the slit diaphragm, which include endocytosis by endothelial cells, uptake by podocytes and entrapment in the paramesangium (Tojo et al., 2008). Moreover, immunogold ultrastructural studies have shown that albumin is taken up by rat podocytes following puromycin aminonucleoside administration (Kinugasa et al., 2011).

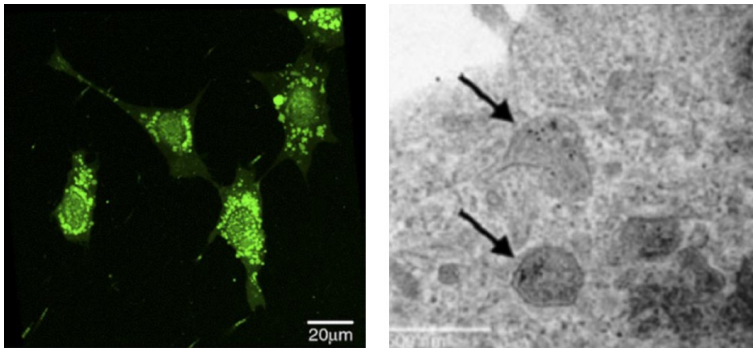


Figure 6: Albumin endocytosis by mouse podocytes *in vitro* and *in vivo*. *The process was visualized by confocal (left) and electron (right) microscopy (Modified from Eyre et al., 2007).*

Using electron microscopy, Chung et al. have shown increased numbers of protein-containing vesicles in podocytes under nephrotic conditions in which there is significant leakage of proteins across the GBM (Chung et al., 2015). In addition, Kinugasa et al. observed an increased endocytosis of albumin by podocytes *in vivo* in a rat model of minimal change disease and that albuminuria was decreased after treatment of proteinuric animals with an antibody that blocked transcytosis (Kinugasa et al., 2011).

Moreover, it was demonstrated that the presence of free fatty acids associated with serum albumin stimulated micropinocytosis in cultured podocytes (Chung et al., 2015), leading to podocyte dysfunction directly or indirectly through activation of angiopoietin-like 4, a protein secreted by podocytes that induces proteinuria (Clement et al., 2011). Furthermore, albumin exposure at levels comparable to what is found in the urine of patients with nephrotic syndrome increased cell death, pro-inflammatory cytokines and pro-apoptotic pathways in a cultured podocyte-like cell line, directly eliciting injury (Agrawal et al., 2014; Okamura et al., 2013).

Evidences are emerging that podocytes are able to endocytose proteins such as albumin using kinetics consistent with a receptor-mediated process. Pawluczyk et al. demonstrated that receptor-mediated endocytosis of albumin by podocytes was regulated by the fatty acid moiety, although some of the detrimental effects were induced independently of it, but the receptor responsible for uptake was not CD36 (Pawluczyk et al., 2014). Previously, it was demonstrated that human podocytes express megalin and cubilin both *in vivo* and *in vitro* and ClC-5 *in vivo* (Prabakaran et al., 2011; Prabakaran et al., 2012; Ceol et al., 2012). Recently, it was confirmed the existence of Ang II-mediated albumin endocytosis by rat podocytes involving a megalin dependent process (Schießl et al., 2016). Furthermore, rat podocytes may degrade albumin in lysosome-like acidified vesicles as it was already demonstrated in cultured human urine-derived podocyte-like epithelial cells (Schießl et al, 2016; Carson et al., 2014). Moreover, Shank2, a scaffolding protein that binds proteins involved in modulating actin dynamics and endocytosis regulation, was also

demonstrated to be required in albumin endocytosis through caveolae by podocytes, subsequently degraded by lysosomes (Dobrinskikh et al., 2014; Dobrinskikh et al., 2015; Carson et al., 2014). Beyond the mechanism of albumin endocytosis, recent immunogold-tracing electron microscopy and time-lapse fluorescent microscopy experiments suggest that podocytes endocytosis may regulate recycling of nephrin giving an important role for this mechanism in podocyte slit diaphragm regulation (Inoue and Ishibe, 2015).

2.2.3. Endocytosis in PECs

Podocytes are not the only cell type involved in albumin uptake at glomerular level. Recently, the Shankland group proposed the hypothesis that an important function of PECs was to restrict protein in the glomerular ultrafiltrate to the confines of Bowman's space, thereby preventing protein from passing into the extraglomerular space (Ohse et al., 2009a). Moreover, they described a model in which whenever there was increased permeability of the glomerular filtration barrier (due to injury to either the endothelial cell, GBM, and/or podocyte), the increased filtered proteins were handled by various mechanisms, including not only the uptake by proximal tubular cells but also by podocytes and PECs. This mechanism leaded to an inflammatory response, recognized as periglomerular inflammation and ultimately fibrosis (Ohse et al., 2009a). In 2012, the same group showed that PECs were able to internalize albumin in normal and overload conditions. Furthermore, they observed an increase of the apoptotic process in the presence of an excess of protein uptake, process which was ameliorated by increasing ERK1/2 phosphorylation (Chang et al., 2012).

Recently, albumin was identified as a signaling molecule that can stimulate MMP-9 production by activated glomerular parietal cells, which may play an important role in PECs migration and podocyte dysfunction during the development and progression of diabetic nephropathy (Zhang et al., 2015). Nevertheless, the mechanisms involved in albumin handling and whether this eventually leads to proliferation, or transdifferentiation remains to be delineated (Ohse et al., 2009b).

2.3. ENDOCYTOSIS IN PROXIMAL TUBULE

Protein endocytosis at tubular level needs an active receptor-mediated pathway that principally involves megalin (LRP2), cubilin, Amnionless (AMN), Disabled-2 (Dab2) and ClC-5 (Figure 7). Megalin and the cubilin-AMN complex (CUBAM) are the main actors in protein uptake mechanism since both megalin and cubilin present albumin binding

sites in their structure. ARH and Dab2 are involved in the vesicle trafficking binding to the NPXY motif present in both megalin and AMN but interacting with different adaptor proteins (ARH binds to microtubules via dynein while Dab2 binds to actin filaments via myosin VI). Although a small proportion of ClC-5 can be detected in the apical brush-border membrane of renal proximal tubular cells, its importance in the endocytic process was well described (Gianesello et al., in press).

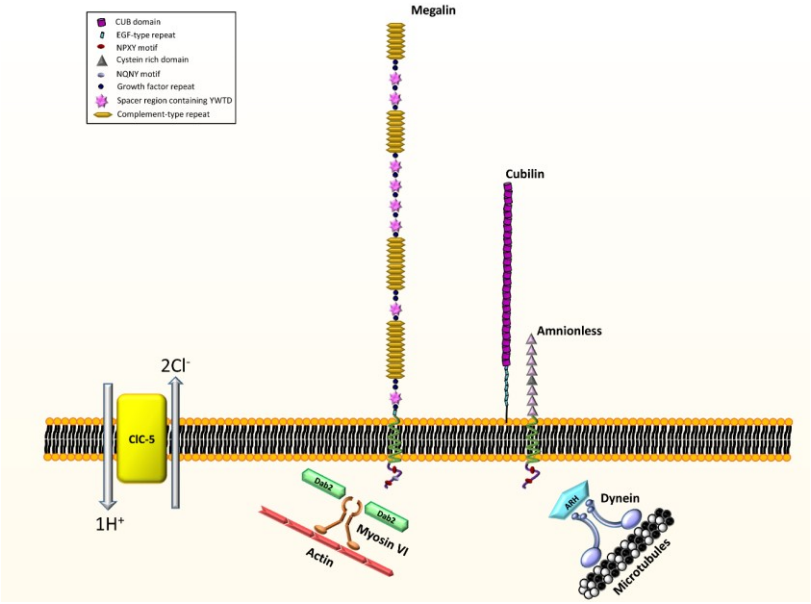


Figure 7. The protein uptake system in proximal tubule epithelial cells. *Diagrammatic representation of the tubular uptake machinery (Gianesello et al., in press).*

2.3.1. Megalin (*LRP2*)

Megalin (gp330) was first identified as a rat Heymann nephritis antigen (Kerjaschki and Farquhar, 1982). Cloning and sequencing of the megalin encoding gene *LRP2* uncovered a giant, glycosylated protein (600 kDa, 4,655 amino acids) with similarities to endocytic receptors of the LDL receptor family (Saito et al., 1994).

As a common characteristic of the LDL receptor superfamily, megalin possess a large extracellular domain followed by a single transmembrane region and a cytoplasmic domain that contains two highly conserved endocytic motifs (NPXY) and an NPXY-like motif (NQNY), which is involved in the apical sorting of megalin (Takeda et al., 2003). The two NPXY motifs sequester protein complexes consisting of, among others, clathrin, clathrin adaptor 2 (AP-2), Dab2, and ARH, which are involved in coated pit formation (Nagai et al., 2005; Schmid et al., 2006; Shah et al., 2013) (Figure 7).

Megalin plays a particular key role in the proximal tubular uptake of glomerular-filtered albumin and other low molecular weight (LMW) proteins. As previously described (Table 1), several physiologically important substrates have been identified as megalin ligands including insulin, albumin, hemoglobin, vitamin D-binding protein

(DBP), retinol-binding protein (RBP), β_2-microglobulin etc. In addition, a number of toxic substances, such as glycated proteins (AGEs), myeloma light chain, and amino glycosides, also interact with megalin and undergo endocytosis, leading to PTEC injury (Christensen et al., 2009). In the kidney, megalin is highly expressed in clathrin-coated pits and is also expressed at lower levels in proximal tubular epithelia cells microvilli (Christensen et al., 2009). A recent study revealed that human podocytes express megalin both *in vivo* and *in vitro* (Prabakaran et al., 2011) (Figure 8).

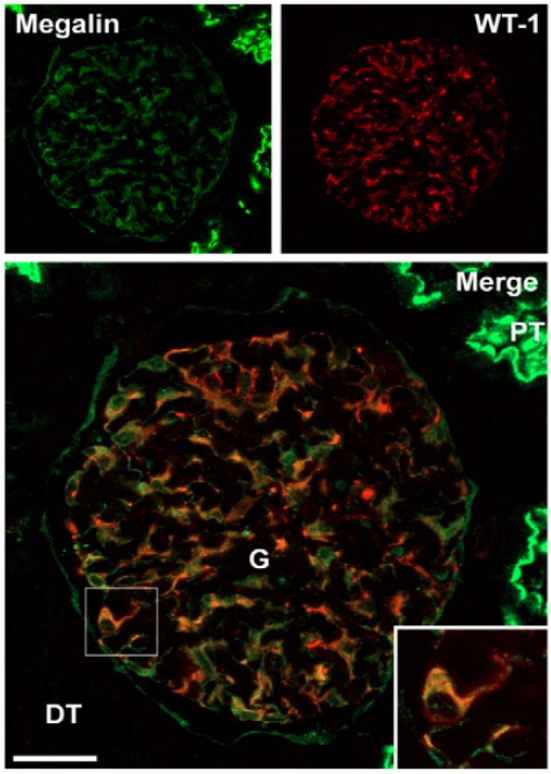


Figure 8. Megalin expression *in vivo*. *Megalin expression in human tubuli and glomeruli. The yellow/orange signal identifies the co-labeling between megalin and WT-1, a podocyte marker (Modified from Prabakaran et al., 2011).*

Mutations of the *LRP2* gene are associated with Donnai-Barrow syndrome (DBS), which is characterized by facio-oculo-acoustico-renal syndrome, LMW proteinuria and albuminuria, agenesis of the corpus callosum, congenital diaphragmatic hernia, facial dysmorphology, ocular anomalies, hearing loss, and developmental delay (Kantarci et al., 2007). Normal renal function was reported in most DBS patients; however, there have been reports of a progressive decline in estimated glomerular filtration rate (Storm et al., 2013a) associated with evidence of focal glomerulosclerosis, which has also been observed in other patients (Shaheen et al., 2010).

Megalin knockout (KO) mice develop LMW proteinuria and albuminuria. Moreover, they have important ultrastructural changes in the endosomal compartments of

proximal tubular epithelial cells, including the absence of apical dense tubules, which correspond to the apical recycling compartment, and other endocytic structures such as clathrin-coated pits and vesicles (Leheste et al., 1999). Constitutive KO of megalin caused perinatal death in the majority (approximately 98%) of homozygous megalin KO mice due to respiratory insufficiency. These mice exhibited forebrain defects and a decreased proximal tubular endocytic apparatus, whereas overall kidney structure was unaffected (Leheste et al., 1999; Christensen and Willnow, 1999). These findings demonstrated the essential role of the receptor in murine lung and forebrain organogenesis consistent with the localization of megalin in developing lung and brain, while the absence of the receptor during early nephron development did not cause obvious renal malformations, except for the decreased endocytic system in the proximal tubule (Nielsen et al., 2016). In megalin-deficient zebrafish an attenuation of endocytic activity and of the associated cellular machinery of the pronephros was noted, with no change in the expression of glomerular or tubular cell markers (Kur et al., 2011, McMahon et al., 2014). In mice, the renal phenotype is associated with functional defects including LMW proteinuria, and the receptor has been shown to be necessary for endocytosis of DBP and the subsequent proximal tubule activation of vitamin D (Leheste et al., 1999; Nykjær et al., 1999; Leheste et al., 2003). Besides proteinuria, changes in renal function have not been reported in megalin KO mice, but it may be that despite the normal overall renal structure in megalin KO mice and DBS patients, the absence of megalin during nephrogenesis compromises renal development, for instance in terms of nephron numbers, manifesting as renal decline later in life. Alternatively, megalin may be important for the maintenance of normal podocyte endocytic activity that is important in podocyte function, or for the clearance of filtered proteins captured in the glomerular basement membrane (Soda and Ishibe, 2013).

2.3.2. Cubilin (*CUBN*)

Cubilin, which was first identified as the receptor for intrinsic factor-vitamin B12, is co-expressed with megalin on apical tubular epithelial cells (Seetharam et al., 1988; Seetharam et al., 1997) (Figure 7). Moreover, a recent study revealed that human podocytes express cubilin both *in vivo* and *in vitro* (Prabakaran et al., 2012) (Figure 9).

This huge protein (glycosylated 460 kDa), shares no homology with other known receptors (Seetharam et al., 1988; Seetharam et al., 1997). Cubilin ligands may be divided into ligands that bind only to cubilin and ligands that bind to both megalin and cubilin. Whereas some vitamin carriers such as RBP apparently binds exclusively to megalin, others such as DBP binds with similar affinity to both megalin and cubilin, and albumin probably with highest affinity to cubilin (Christensen et al., 2012) (Table

1). This peripheral membrane glycoprotein lacks a transmembrane as well as intracellular domain, but anchored *via* its NH_2 terminus to apical membranes in proximal tubular epithelial cells.

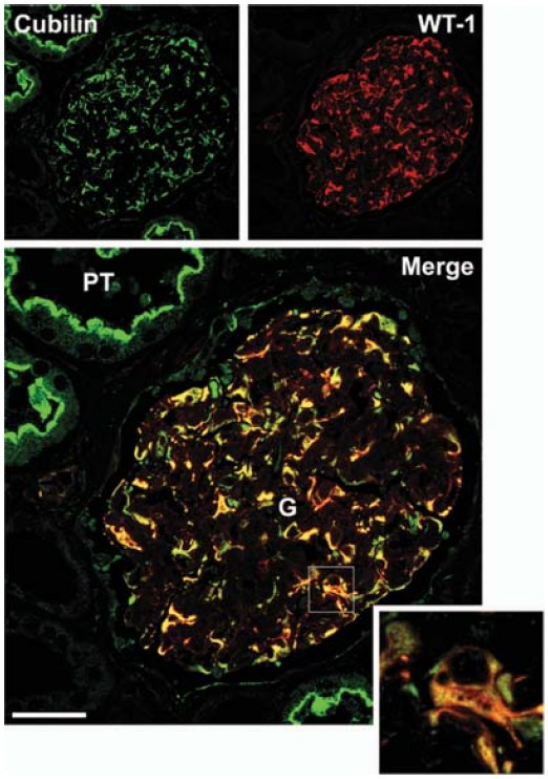


Figure 9. Cubilin expression *in vivo*. *Cubilin expression in human tubuli and glomeruli. The yellow/orange signal identifies the co-labeling between cubilin and WT-1, a podocyte marker (Prabakaran et al., 2012).*

As cubilin lacks an intracellular domain, it is thought to require interaction with megalin for proper functioning. However, cubilin forms a functional complex with amnionless (AMN) named CUBAM, which is translocated to the plasma membrane and displays megalin-independent activity. Moreover, cubilin is dependent on AMN for its normal translocation from the endoplasmic reticulum to the membrane as well as for consequent endocytosis (Fyfe eta al., 2004; Coudroy et al., 2005).

Serving as an endocytic receptor, cubilin mediates the uptake of proteins and protein-bound substances both in the intestine and in the kidneys. In the kidney, proximal tubule cubilin mediates the uptake of several filtered proteins and the combined action of cubilin and megalin essentially clears the ultrafiltrate of filtered proteins, lipids, vitamins and hormones resulting in an almost protein-free final urine. Albumin was among the first ligands to be identified showing the importance of cubilin for urinary albumin excretion (Birn et al., 2000; Zhai et al., 2000).

Mutations in the *CUBN* gene cause hereditary megaloblastic anaemia 1 and Imerslund-Gräsbeck syndrome (IGS), which are associated with selective vitamin B_{12} malabsorption and proteinuria (Aminoff et al., 1999). The LMW proteinuria in most IGS patients is consistent with defective proximal tubule reabsorption and with observations in cubilin-deficient mice (Storm et al., 2011; Storm et al., 2013b; Wahlstedt-Fröberg et al., 2003).

The first attempt to produce cubilin inactivation by a gene-targeted approach showed an important role of cubilin during murine development, possibly being involved in maternal-to-embryo transport (Smith et al., 2006). Studies in mice with kidney-specific *Cubn* gene knockout confirmed that cubilin is essential to normal tubular uptake of albumin, supported by a dramatically increased urinary albumin excretion in the single cubilin knockout mode (Amsellem et al., 2010). These data were consistent with observations in dogs with cubilin dysfunction (Birn et al., 2000). Another model of cubilin deletion also resulted in albuminuria, indicating that cubilin is the major albumin binding receptor (Weyer et al., 2011). However, when both *Lrp2* and *Cubn* were deleted, the Authors revealed a greater albumin excretion than mice deficient in cubilin alone, indicating an independent role of megalin as well (Weyer et al., 2011).

Observations suggested some additional differences between human and mouse kidneys in the relative importance of megalin and cubilin for the uptake of ligands (Nielsen et al., 2016). No increase in urinary DBP excretion is observed in cubilin deficient mice indicating that, in mice, functional megalin is sufficient for normal tubular reabsorption of DBP (Amsellem et al., 31). In humans, however, urinary excretion of DBP is increased in both megalin-deficient and cubilin-deficient patients indicating that complete, tubular reabsorption of DBP depends on both megalin and cubilin (Storm et al., 2011; Storm et al., 2013a).

2.3.3. Amnionless (AMN)

Amnionless (AMN) exists in at least five different sizes ranging from 38−50 kDa. Cubilin and AMN co-localize closely in the kidney, intestine and yolk sac of mice and AMN is essential during biosynthesis and trafficking of cubilin (Kalantry et al., 2001; Tanner et al., 2003; Fyfe et al., 2004) (Figure 7). The association to cubilin occurs through the epidermal growth-factor-like repeats in cubilin (Coudroy et al., 2005). It is difficult to exclude whether AMN contributes to cubilin endocytosis also when cubilin is associated to megalin, since studies of cubilin without AMN are difficult to perform due the interdependent apical sorting of AMN and cubilin (Christensen et al., 2012). In cell cultures lacking megalin, cubilin is endocytosed, probably relying on the endocytic properties of AMN (Kalantry et al., 2001; Fyfe et al., 2004).

At tubular level, efficient uptake of filtered proteins requires a high-capacity endocytic system and in this setting the CUBAM complex is unable to reabsorb filtered cubilin ligands in the absence of megalin as evidenced both in the *Lrp2* knockout mice and in megalin-deficient patients (Ansellem et al., 2010; Storm et al., 2013a). These observations indicated that the molecular mechanisms for intestinal absorption of intrinsic factor-B_{12} are different from the proximal tubular reabsorption of protein. This does not exclude that AMN is able to mediate endocytosis of cubilin independently of megalin in the proximal tubule, as observed when Intrinsic Factor B_{12} is injected in megalin-deficient mice; however, the uptake is significantly reduced (Ansellem et al., 2010).

Imerslund-Gräsbeck syndrome (IGS) is a rare autosomal-recessive disease due to mutations of *AMN* or *CUBN* genes. The disease is characterized by reduced absorption of intrinsic factor-B_{12} in the small intestine. Proteinuria varies greatly between patients but is due to reduced function of cubilin/AMN in the proximal tubule (Wahlstedt-Fröberg et al., 2003). Giant Schnauzer dogs with inherited, selective vitamin B_{12} malabsorption were the first reported model of cubilin dysfunction. This inherited condition was shown to be caused by a spontaneous gene defect in the *AMN* gene (Fyfe et al., 1991; Xu et al., 1999).

2.3.4. Disabled 2 (Dab2)

Disabled-2 (Dab2, also known as DOC-2 or p96) was described as a member of cargo-specific adaptor proteins as it binds to megalin, clathrin, AP-2, and phosphoinositides (Koral and Erkan, 2012).

Dab2 binds to the cytosolic FXNPXY domain of members of the LDL receptor family (as megalin) *via* its N-terminal domain (Morris and Cooper, 2001); to the motor protein myosin VI (Figure 7), implicated in endocytosis, *via* the C-terminal domain, and to clathrin and AP-2 *via* two motifs of its central region. All these interactions endow Dab2 the role of coordinating vesicle coat assembly, cargo recruitment, vesicle formation and vesicle trafficking (Vázquez-Carretero et al., 2014).

In the kidney, Dab2 acts as an adaptor protein in the megalin-mediated endocytosis of filtered proteins (Vázquez-Carretero et al., 2014). The adaptor protein Dab2 and megalin mutually regulate each other's localization in PTECs. The expression of Dab2 in PTECs appears to be dependent on megalin or factors associated with megalin, while knocking-out of the *Dab2* gene decreases the level and alters the subcellular distribution of megalin in PTECs (Nagai et al., 2005). Dab2 is not only crucial for megalin trafficking, but also for endocytosis of the endocytic receptor CUBAM since AMN provides the two NPXY signals that give the potential endocytic capacity *via* Dab2 (Pedersen et al., 2010).

Dab2 is a very versatile protein because of its ability to convey internalization, sorting, differentiation, and proliferation signals. The role of Dab2 in cell signaling events has been shown in a number of systems (Koral and Erkan, 2012). The *DAB2* gene is implicated in cell growth control and it is downregulated in many human carcinomas, suggesting that continued expression is incompatible with tumor development (Morris and Cooper, 2001).

2.3.5. ClC-5

In the human kidney, ClC-5 is primarily expressed in proximal tubular cells, in cortical collecting duct α intercalated cells and in the thick ascending limb of Henle's loop. In proximal cells, it is predominantly located in intracellular subapical endosomes, which are involved in the endocytic reabsorption of LMW proteins that have passed the glomerular filter. ClC-5 was firstly proposed to provide a shunt conductance in early endosomes permitting an efficient intraluminal acidification by V-type H^+-ATPase (Günther et al., 1998; Luyckx et al., 1998; Devuyst et al., 1999; Hara-Chikuma et al.; 2005). However, two independent groups demonstrated that ClC-5 functions as a Cl^-/H^+ antiporter when activated by positive voltages (Picollo and Pusch, 2005; Scheel et al., 2005). A small portion of the ClC-5 channels is also located on the cell surface, where it is thought to mediate plasma membrane chloride currents (Günther et al., 1998; Luyckx et al., 1998; Devuyst et al., 1999; Friedrich et al., 1999), or to participate in the macromolecular complexes at plasma membrane level deputed to LMW proteins and albumin endocytosis (Hryciw et al., 2006) (Figure 7).

Recently, it was demonstrated that ClC-5 is expressed in human glomeruli of normal and proteinuric kidneys, in particular in podocytes. Moreover, ClC-5 was found to be overexpressed in glomeruli of diabetic nephropathy and membranous glomerulonephritis patients both at mRNA and protein level suggesting a role for this protein in albumin endocytosis by podocytes (Ceol et al., 2012) (Figure 10).

Mutations in the *CLCN5* gene are the main cause of Dent's Disease (Jentsch et al., 1995; Thakker, 1997; Waldegger and Jentsch, 2000). The term Dent's disease was first introduced in 1990 (Wrong et al., 1990) and identifies a group of X-linked renal disorders characterized by LMW proteinuria and variable presence of hypercalciuria, nephrocalcinosis and/or nephrolithiasis (Lloyd et al., 1996; Thakker, 2000). Growing number of reports describe patients with Dent's disease with nephrotic-range proteinuria, histological findings of Focal Segmental and/or global Glomerulosclerosis (FSGS) and episodic evidence of isolated proximal tubular dysfunction (Copelovitch et al., 2007; Frishberg et al., 2009; Kaneko et al., 2010; Cramer et al., 2014; De Mutiis et al., 2015). Moreover, Dent's disease patients display a significant reduction of cubilin and megalin expression in proximal tubular epithelial cells, probably as a

consequence of a disturbed intracellular trafficking of the receptors (Christensen et al., 2013).

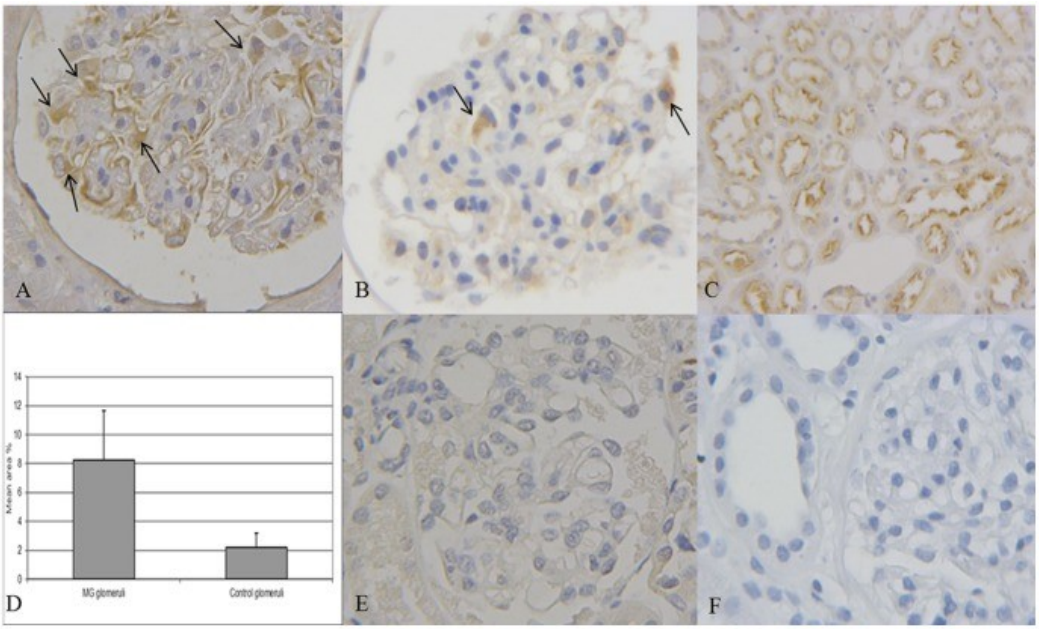


Figure 10. Immunoistochemical analysis in Membranous glomerulonephritis (MG) biopsies. *A. MG patient (400X). B. Control glomerulus (400X). C. Proximal tubular cells used as internal control (200X). D. ClC-5 quantification by morphometric analysis on MG and control glomeruli. ClC-5 signal was significantly higher in MG than in control samples (p<0.01). E. Dent's disease patient (400X). F. Negative control obtained omitting primary antibody (400X) (Ceol et al., 2012).*

2.4. PROTEINURIC NEPHROPATHIES

2.4.1. Proteinuria

Damage to the glomerular filtration barrier causes protein to leak into the glomerular filtrate, which results in abnormally high concentrations in the urine: this is known as proteinuria. Proteinuria can itself cause injury, which is mediated either by the properties of specific proteins in the filtrate or simply through the mass of filtered protein. Increased protein in tubular fluid enhances reabsorption by the tubular epithelial cells and can overload their catabolic capacity, which results in a lysosomal burst and in the release of cathepsins into the cytoplasm (Kurts et al., 2013).

Proteinuria is a clinically important sign of early renal dysfunction (Reiser and Altinas, 2016). Proteinuria is said to be present when the urine contains more than 300 mg protein per day (or 200 mg/l). In the nephrotic syndrome, more than 3.5 g/day of proteins are excreted in the urine. Urine with daily protein excretion of 30–300 mg (20–200 mg/l) reflects microalbuminuria (Haraldsson et al., 2008).

Proteins in the final urine have different origins. First, proteins cross the glomerular barrier, and tubular cell reabsorption modifies their final concentration. Second,

there is tubular secretion of proteins from the blood. Third, proteins may be synthesized by the cells themselves and released into the urine. Fourth, the proteins may be added to the urine at a later stage (e.g., by excretion from the prostate gland in men) (Haraldsson et al., 2008).

Albuminuria is a powerful independent predictor of progression of renal disease, cardiovascular disease and death in people with renal disease, hypertension, diabetes, vascular disease, and in the general population (Schmieder et al., 2011). Early detection and prevention of kidney disease is the only way to prevent associated morbidity and premature mortality from cardiovascular disease or end stage renal disease; however, kidney disease is often asymptomatic until advanced stages, making early detection unlikely in the absence of active surveillance, and this limits the opportunity for treating and delaying disease progression (Clark et al., 2011). It was demonstrated that in a population characterized by an increase of albuminuria due to the progressive nature of cardiovascular disease, changes in albuminuria are indicative of a worse prognosis, and serial measurements of albuminuria characterize changes in cardiovascular and renal prognosis and all-cause mortality (Schmieder et al., 2011).

Proteinuria may occur regardless of whether the damage is at glomerular (podocytes, GBM or endothelial cells) (Haraldsson et al., 2008) or tubular (Christensen et al., 2009) level. The proximal tubule is appointee to retrieve LMW proteins from the glomerular filtrate. Defects in the receptors that mediate the uptake of these filtered ligands or saturation of the clearance pathway (e.g., in diabetes), lead to LMW proteinuria. Prolonged LMW proteinuria (also referred to as tubular proteinuria because it does not involve glomerular dysfunction) causes further deterioration in kidney function and leads to renal failure (Raghavan and Weisz, 2015).

2.4.2. Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by loss of tolerance against nuclear autoantigens, lymphoproliferation, polyclonal autoantibody production, immune complex disease, and multiorgan tissue inflammation (Lech and Anders, 2013). Clinically, it is an unpredictable, remitting and relapsing disease that may involve virtually any organ in the body; however, it affects mainly the skin, kidneys, serosal membranes, joints, and heart. Immunologically, the disease is related with many autoantibodies, classically including antinuclear antibodies (ANAs). Although the kidney appears normal by light microscopy in 25% to 30% of cases, almost all cases of SLE show some renal abnormality if examined by immunofluorescence and electron microscopy (Kumar et al., 2013).

The link between SLE and lupus nephritis is the production of autoantibodies that bind to autoantigens in the kidneys; for example, a subset of double-stranded DNA (dsDNA)-specific antibodies cross-react with annexin II on the cell surface, in the cytoplasm and in the nucleus of mesangial cells, and also cross-react with nucleosomes in the mesangium and in the glomerular capillary epithelium. The extent and the progression of glomerular immunopathology depends on the site of immune complex formation, as this determines the predominant glomerular cell type that is affected (Kurts et al., 2013).

Immune deposits can be found in the mesangium, subendothelium, or subepithelium. Subendothelial deposits are in contact with the vascular space and can recruit myeloid cells into the renal parenchyma. Subepithelial cells are in contact with podocytes and the urinary space. Podocytes and endothelial cells communicate with each other by transfer of soluble growth factors across the glomerular basement membrane, whereas mesangial cells and endothelial cells can interact with each other directly *via* receptors and their ligands (Davidson, 2016). *In situ* formation of immune complexes with complement activation and induction of inflammatory mediators results in chemotaxis and activation of inflammatory cells within the kidney and further release of proinflammatory signals and mediators, leading to injury and damage of cellular and structural elements within the renal parenchyma (Chan, 2015) (Figure 11).

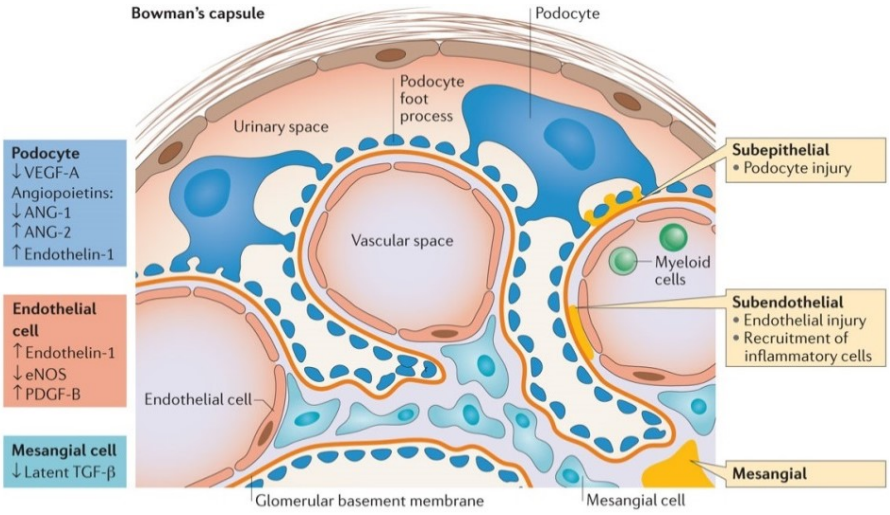


Figure 11. Glomerular injury in lupus nephritis. *Immune deposits can be found in the mesangium, subendothelium, or subepithelium. Alterations of soluble growth factors in lupus nephritis are shown by the arrows and colour-coded according to cell type (Davidson, 2016).*

According to the current International Society of Nephrology/Renal Pathology Society (ISN/RPS) morphologic classification revised in 2004, there are six patterns of glomerular disease in SLE (none of which is specific to the disease) (Table 2) (Weening et al., 2004).

Immune complexes can form in different parts of the glomerulus, which determines the resulting histopathological lesion, as different glomerular cell types are primarily activated in each compartment. Immune complex deposition in the mesangium activates mesangial cells, which leads to mesangioproliferative glomerulopathies, such as lupus nephritis class I and II. Subendothelial immune complex deposits activate endothelial cells, as seen in lupus nephritis class III and IV. Subepithelial immune complex deposits preferentially activate the visceral glomerular epithelium — that is, podocytes — and usually cause massive proteinuria. As a result of the poor regeneration of podocytes compared with that of the other glomerular cell types, podocyte loss leads to progressive membranous nephropathy and end-stage renal disease. Hence, the level of proteinuria is an important prognostic biomarker and predictor of poor outcomes of glomerulopathies (Kurts et al., 2013).

Class I	**Minimal mesangial lupus nephritis** Normal glomeruli by light microscopy, but mesangial immune deposits by immunofluorescence
Class II	**Mesangial proliferative lupus nephritis** Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits There may be a few isolated subepithelial or subendothelial deposits visible by immunofluorescence or electron microscopy, but not by light microscopy
Class III	**Focal lupus nephritis[a,b]** Active or inactive focal, segmental or global endo- or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations
Class III (A)	Active lesions: focal proliferative lupus nephritis
Class III (A/C)	Active and chronic lesions: focal proliferative and sclerosing lupus nephritis
Class III (C)	Chronic inactive lesions with glomerular scars: focal sclerosing lupus nephritis
Class IV	**Diffuse lupus nephritis[a,b]** Active or inactive diffuse, segmental or global endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental (IV-S) lupus nephritis when ≥50% of the involved glomeruli have segmental lesions, and diffuse global (IV-G) lupus nephritis when ≥50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation
Class IV-S (A)	Active lesions: diffuse segmental proliferative lupus nephritis
Class IV-G (A)	Active lesions: diffuse global proliferative lupus nephritis
Class IV-S (A/C)	Active and chronic lesions: diffuse segmental proliferative and sclerosing lupus nephritis
Class IV-G (A/C)	Active and chronic lesions: diffuse global proliferative and sclerosing lupus nephritis
Class IV-S (C)	Chronic inactive lesions with scars: diffuse segmental sclerosing lupus nephritis
Class IV-G (C)	Chronic inactive lesions with scars: diffuse global sclerosing lupus nephritis
Class V	**Membranous lupus nephritis** Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations Class V lupus nephritis may occur in combination with class III or IV in which case both will be diagnosed Class V lupus nephritis may show advanced sclerosis
Class VI	**Advanced sclerosing lupus nephritis** ≥90% of glomeruli globally sclerosed without residual activity

[a] Indicate the proportion of glomeruli with active and with sclerotic lesions.
[b] Indicate the proportion of glomeruli with fibrinoid necrosis and with cellular crescents.
Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.

Table 2. International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis. *(Weening et al., 2004).*

2.4.3. Minimal Change Disease (MCD)

Minimal change disease (MCD) is the most common disease underlying childhood nephrotic syndrome, but also manifests in adults. Secondary forms occur in diseases such as atopy or malignancy, particularly in lymphomas (Floege and Amann, 2016). MCD is characterized by massive proteinuria and hypoalbuminemia, resulting in edema and hypercholesterolemia. Histological findings of the disease in glomeruli are typically normal by light microscopy and only electron microscopy shows effacement of podocyte foot processes without electron-dense immune deposits. These manifestations are typically reversible with the use of corticosteroid therapy in steroid-sensitive nephrotic syndrome, so that progressive loss of renal function is rare (Saleem and Kobayashi, 2016).

Whether MCD and focal segmental glomerulosclerosis (FSGS) represent different manifestations of one disease (with MCD potentially progressing to FSGS) or two different diseases is unknown. FSGS is a confusing term because it is used by pathologists both to describe a glomerular scar, which can result from almost any injury affecting the kidneys, and clinically to denote a family of glomerular diseases.

Central to the pathogenesis of FSGS is the podocytes damage, resulting in their loss (Floege and Amann, 2016). A second central event is the activation of parietal epithelial cells on Bowman's capsule, which migrate onto the glomerular tuft to replace or displace podocytes. Once on the glomerular tuft, parietal cells, unlike podocytes, are unable to produce sufficient vascular endothelial growth factor, and this triggers endothelial problems, with collapse and scarring of the affected capillary. The finding of parietal cell activation markers can help to distinguish focal segmental glomerulosclerosis from minimal change disease (Floege and Amann, 2016).

3. Aims

Mechanisms of glomerular protein handling have received much attention in the study of nephrotic syndrome, and proteinuria has been recognized as an independent risk factor for both renal failure and cardiovascular disease.

Receptor-mediated endocytosis is a process by which cells can selectively and actively internalize biological substances. It is tightly regulated by intracellular mechanisms involving pathways which are common to many different cell types. In the kidney, proximal tubular epithelial cells (PTECs) are the main actors in protein uptake. This mechanism requires the coordinated functioning of megalin, cubilin, AMN, Dab2 and ClC-5 to perform albumin endocytosis.

Very recent studies have demonstrated the presence of ClC-5, cubilin and megalin at glomerular level. Furthermore, there is a clear evidence of the presence of endocytic vesicles in podocytes.

Aims of this project were:

i. To confirm human podocyte ability to internalize albumin *in vitro*;

ii. To verify the presence of ClC-5, megalin, cubilin, Dab2 and AMN in human podocytes *in vitro* and whether albumin overload could be able to modulate their expression;

iii. To investigate ClC-5, megalin and cubilin expression in biopsies of patients with lupus nephritis characterized by different levels of proteinuria;

iv. To recognize which cells are involved in the expression of ClC-5, megalin and cubilin in the glomerular compartment in kidney biopsies of SLE patients.

4. Methods

4.1. HUMAN PODOCYTE CELL CULTURE

Human podocytes have been kindly donated by Prof. Saleem from the University of Bristol who established cell culture conditions that allow the differentiation of podocytes *in vitro* (Saleem et al., 2002). Human conditionally immortalized podocytes, primarily cloned from human glomerular cultures, were maintained in RPMI 1640 (Euroclone) supplemented with 10 % fetal bovine serum (FBS; Sigma-Aldrich), Insulin-Transferrin-Selenium supplement (ITS; Sigma-Aldrich), 2 mM L-Glutamine and antibiotics mixture (Euroclone). Fresh media was supplied once every 2 - 3 days.

To stimulate human podocyte proliferation, cells were cultivated at 33 °C in 5 % CO_2 (permissive conditions) to induce expression of temperature-sensitive large T antigens. To induce differentiation, podocytes were maintained at 37 °C in 5 % CO_2 (non-permissive conditions) for at least 2 weeks, and for subcultures 0.05 % trypsin was used to detach cells from the culture dishes. The end of the differentiation process was assessed by podocin positivity, a well-known component of the slit diaphragm. Cell density was kept below 90 % to allow differentiation. Cells were used between passages 7 and 12.

4.2. KIDNEY BIOPSIES

Biopsies of patients affected by Systemic lupus erythematosus (SLE) (n. 23) were obtained for diagnostic purposes. Control biopsies (n. 6) were pieces from site remote from tumor-bearing tissue and were histologically evaluated to disclose a normal morphology. One biopsy of nephrotic syndrome (uProt: 5.81 g/day) presenting Minimal Change Disease (MCD) was also analyzed.

Clinical data at the time of biopsy (proteinuria, pharmacological therapy and Lupus nephritis classification) were collected for all SLE patients (Table 3). All subjects gave their informed written consent to the study, which have been approved by the local Ethical Committee.

Pts	Sex	uProt (g/die)	Class	ACEi/ARB	IT
1	F	4.20	N/A	YES	NO
2	M	4.00	V	NO	YES
3	F	5.08	IV	YES	NO
4	M	10.00	IV-V	YES	NO
5	F	5.00	IV	YES	YES
6	M	1.48	IV	NO	YES
7	M	1.75	III	NO	NO
8	F	8.10	V	YES	NO
9	F	1.50	III	NO	YES
10	F	1.00	IV	NO	NO
11	F	2.63	IV	NO	YES
12	F	2.00	IV	YES	YES
13	F	3.00	IV	NO	YES
14	M	3.78	IV	YES	YES
15	F	1.50	II	NO	NO
16	F	0.23	IV-V	YES	YES
17	F	0.61	IV	NO	NO
18	F	2.00	III	YES	YES
19	M	3.63	IV	YES	YES
20	F	7.46	IV	NO	YES
21	F	3.00	IV-V	NO	YES
22	F	3.66	II	NO	YES
23	F	0.56	IV	NO	NO

Table 3. Characteristics of SLE patients enrolled for this study. *Pts: patients, uProt: urinary proteinuria, N/A: not available, ACEi/ARB: Therapy with ACE inhibitors or ARB, IT: Immunosuppressive therapy.*

4.3. TIME-LAPSE FLUORESCENCE IMAGING

To monitoring BSA endocytosis in real time, podocytes seeded in 24 well-plate were cultured in serum free media for 24 hours. Then, cells were incubated overnight with FITC-BSA 10 µg/ml in medium without phenol red. Images were acquired every 3 minutes. At least 15 cells for each magnification were analyzed to evaluate internalization timing. Time-lapse images were obtained using LAS X Software (Leica Microsystems).

All acquisitions were performed with an inverted fluorescence microscope (DMI600CS-TCS SP8, Leica Microsystems) placed within a home-built temperature

controlled enclosure set at 37 °C and 5 % CO_2 for live cell imaging. Experiments were done twice and acquired at 200X magnification.

4.4. ENDOCYTIC UPTAKE ANALYSIS

Endocytosis quantification was performed as described by Coffey et al (Coffey et al, 2015). Briefly, mature podocytes seeded on 6 well plates were changed in serum free media overnight. Cells were then incubated with FITC-BSA (Sigma-Aldrich) 10 µg/ml, 100 µg/ml and 1 mg/ml for 30 minutes and 2 hours at 37°C. To evaluate nonspecific FITC-BSA uptake (albumin incorporation *via* a non-receptor mediated mechanism), cells were incubated with same concentrations of FITC-BSA for 30 minutes and 2 hours at 4 °C.

Cells were than washed, transferred in ice to stop endocytosis, and washed six times with ice-cold PBS. Any bound but not endocytosed albumin was stripped by ice-cold stripping buffer (0.2 M acetic acid and 2 M NaCl). After five washes with PBS, cells were permealized by a detergent solution (Triton X-100, 0.1 % vol/vol in MOPS solution). Intracellular fluorescence was measured in a single-beam fluorimeter (Victor X3, PerkinElmer) at an excitation wavelength of 490 ± 10 nm and emission wavelength of 520 ± 10 nm.

Fluorescence was normalized for the amount of cells as protein quantity. Cell lysates were obtained by a physical procedure (freezing in liquid nitrogen and defrosting in water at 37 °C) into lysis buffer containing protease inhibitor. Protein quantification was performed at 280 nm with a Nanodrop ND-100 spectrophotometer (Celbio). Results were expressed as fluorescence/milligram of protein. Data are shown as the average ± SD of three experiments in triplicate.

4.5. IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) staining on kidney biopsies was performed on formalin-fixed, paraffin-embedded sections, while differentiated podocytes were seeded on 8 well chamber slides and fixed with cold methanol for 5 minutes at room temperature (RT). For kidney biopsies, after deparaffinization and rehydration in an ethanol series, antigens were retrieved by heating in a microwave oven (250 W for 5 min, 500 W for 5 min and 250 W per 5 min) in 5 mM citrate buffer solution pH 6.0.

All specimens were treated with 3 % H_2O_2 in 50 mM phosphate-buffered saline (PBS) (pH 7.4) for 15 min RT to remove endogenous peroxidases and then incubated with 2 % normal goat serum (Sigma-Aldrich) for 30 min RT to prevent nonspecific binding

of the antibody. Samples were incubated with a rabbit antibody against ClC-5 (Sigma-Aldrich, cod. HPA000401) diluted 1:200 in PBS at 4 °C overnight.

Samples were then rinsed with PBS and treated with DakoCytomation EnVision + System-HRP Labeled Polymer anti-rabbit (DAKO Corporation) in a humidified chamber at room temperature for 30 min. Signals were visualized by 3,3-diaminobenzidine-tetrachloride (DAB, DAKO) and sections were counterstained with hematoxylin. The specificity of the immunolabeling was confirmed by incubation without primary antibody. Images were acquired using the light microscope Diaplan (Leitz).

ClC-5 shared about 85 % homology with ClC-3 and ClC-4. The antibody used in IHC analysis recognizes an epitope in common with the three proteins. Our group previously tested its efficiency for ClC-5 performing immunohistochemistry in a biopsy of Dent's disease patient carrying nonsense mutation on *CLCN5*. This experiment allowed us to demonstrate the specificity of ClC-5 antibody since all the glomeruli and the corresponding tubule-interstitium were negative (Ceol et al., 2012) (Figure 10).

4.6. IMMUNOFLUORESCENCE

For the immunofluorescence (IF) analysis, mature podocytes were seeded on 8 well chamberslides and fixed with cold methanol for 5 minutes at RT.

For co-localization experiments, mature podocytes on 96 well plates were changed to serum free media overnight. Subsequently cells were incubated with FITC-albumin (10 µg/ml) for 2, 4, 8 and 24 h. Finally, cells were fixed with cold methanol for 5 minutes at RT.

Formalin-fixed, paraffin-embedded sections of kidney biopsies were treated as follow. After deparaffinization and rehydration in an ethanol series, antigens were retrieved by heating in a microwave oven (250 W for 5 min, 500 W for 5 min and 250 W per 5 min) in 5 mM citrate buffer solution pH 6.0.

Cells and kidney sections were treated with PBS-Triton X 100 (Sigma) 0.4 % for 15 min, followed by blocking with 10 % normal serum (normal donkey serum (AbCam) or normal goat serum (Sigma-Aldrich) depending on secondary antibody) for 30 min at RT. Samples were incubated with primary antibody (Table 4) diluted in BSA 5 % in PBS at 4 °C overnight.

After three rinses (the first one in PBS containing 0.1 % Triton X and the other two in PBS only) cells were incubated with the appropriate fluorescent secondary antibody diluted in BSA 5% in PBS for 1h at RT (Table 5) protecting from light. After washing

again in PBS containing 0.1 % Triton X and PBS, nuclei were counterstained with DAPI (Boehringer) diluted 1:1,000 in PBS for 5 min at room temperature. Negative controls were performed by omitting primary antibody.

Target	Clone	Host	Manufacturer	Code	Dilution
Podocin	H-130	rabbit	Santa Cruz Biotechnology	sc-21009	1:50
megalin	-	rabbit	LS-Bio	LS-B105	1:100
cubilin	-	sheep	R&D Systems	AF3700	1:50 (cells) 1:100 (biopsies)
AMN	H-281	rabbit	Santa Cruz Biotechnology	sc-135178	1:50
Dab2	H-110	rabbit	Santa Cruz Biotechnology	sc-13982	1:50
ANXA3	-	rabbit	Sigma-Aldrich	HPA013398	1:500
CD44	HI44a	mouse	Immunostep	44PU2	1:20
CD24	SN3	mouse	Santa Cruz Biotechnology	sc-19585	1:20

Table 4. Primary antibodies conditions used in IF analyses.

Target	Host	Manufacturer	Code	Conjugation	Dilution
Anti-rabbit	donkey	Santa Cruz Biotechnology	sc-362291	CFL 647	1:100
Anti-sheep	donkey	Santa Cruz Biotechnology	sc-2477	Rhodamine	1:100
Anti-sheep	donkey	Santa Cruz Biotechnology	sc-2476	FITC	1:100
Anti-mouse	goat	ThermoFisher Scientific	A-11001	Alexa 488	1:1,000

Table 5. Secondary antibodies conditions used in IF analyses.

Images were acquired using a DMI600CS-TCS SP8 fluorescence microscope and analyzed using the LAS X software. Co-localization experiments were done in duplicate.

4.7. APOPTOSIS ASSAY

Annexin V FITC staining was evaluated with a commercial kit paired with PI (Affymetrix-eBioscience) following manufacturer's instructions. Briefly, cells treated on T-75 flasks were detached with Trypsin after 24, 48 and 72 hours' BSA stimulation and washed in PBS. After resuspending mature podocytes in binding buffer, 200-500,000 cells/ml were marked with Annexin V-FITC for 10 minutes RT. Cells were then washed with binding buffer and subsequently resuspended in the same buffer with PI.

In early stages of apoptosis, the plasma membrane excludes viability dyes such as propidium iodide (PI), therefore cells which display only Annexin V staining (PI negative) are in early stages of apoptosis. During late-stage apoptosis, loss of cell membrane integrity allows Annexin V binding to cytosolic PS, as well as cell uptake of PI. Apoptotic stages were evaluated by flow cytometry using a CytoFLEX cytometer (Beckman Coulter). Cells without Annexin and PI were used to evaluate gate parameters.

4.8. RNA EXTRACTION, QUANTIFICATION AND CDNA SYNTHESIS

Podocytes were cultured on T-75 flasks. Medium was changed to serum free media overnight before experiments. Cells were incubated with increasing concentration of albumin (BSA; Sigma-Aldrich) (range 10 µg/ml - 30 mg/ml) for 2 - 4 - 8 - 24 - 48 - 72 hours. Control cells were cultured without stimulation.

RNeasy Mini Kit (Qiagen) was used to isolate total RNA from cells, following the spin column protocol. RNA was finally eluted in RNase free water and stored at -80°C. RNA concentration was measured with Nanodrop ND-100 spectrophotometer and purity was checked by the A260/A280 ratio (between 1.8 and 2). RNA integrity was valued by capillary electrophoresis on Agilent RNA Nano chip with Agilent 2100 Bioanalyzer (Agilent Technology), by observing and quantifying integrity of 28S and 18S rRNAs. For Real Time PCR analyses were used only RNA with an RNA integrity number (RIN) at least of 9.

RNA was retro-transcribed from a starting quantity of 100 ng in a final volume of 20 µl. Reaction mix was prepared as follows: 5 mM $MgCl_2$ (Sigma-Aldrich); 1 mM dNTPs (Roche); 2.5 µM random hexamers (EurX); 1 U RNase inhibitor (EurX); 2.5 U MuLV reverse transcriptase (ThermoFisher Scientific) in 50 mM KCl, 10 mM Tris HCl pH 8.3 (Sigma-Aldrich).

Reactions were performed on 2720 thermal cycler (ThermoFisher Scientific) applying the following thermal profile: room temperature for 10 min, 42 °C for 30 min, 65 °C for 5 min, 4 °C for 5 min.

4.9. REAL TIME PCR

Primer pairs for the region of interest were designed according to stringent parameters to ensure successful assays and convenient experiment design by using Primer3 software ver. 4.0 (http://primer3.ut.ee). The NCBI Primer-BLAST program was used for *in silico* specificity analysis (www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). After *in silico* quality control, validation of each primer pair was performed. Microchip electrophoresis on Agilent 2100 BioAnalyzer, Sanger sequencing and melting curve analysis were used to check the specificity of PCR reactions. Amplification curves for all primers were established and resulted in at least 85% in efficiency. An overview of the primers used is given below in Table 6.

Name	NCBI Reference Sequence	Sequence
GLIC For	NM_17851.1	GAAGGTGAAGGTCGGAGT
GLIC Rev		TGGCAACAATATCCACTTTACCA
B2M For	NM_004048.2	TCTCTCTTTCTGGCCTGGAG
B2M Rev		TCTCTGCTGGATGACGTGAG
HPRT1 For	NM_000194.2	CCTGGCGTCGTGATTAGTGA
HPRT1 Rev		TCTCGAGCAAGACGTTCAGT
CLCN5 For	NM_000084.4	TGCTGGAACTCTGAGCATGT
CLCN5 Rev		TACGGCAAGGAAGGCAAATA
CUBN For	NM_001081.3	GCCGTGAGAAAGGATTTCAG
CUBN Rev		TCCTTGTTTGGTGGATACCTG
LRP2 For	NM_004525.2	TGGGTTGACTCTCGGTTTGA
LRP2 Rev		CACGGCCATCTTTGTCCAAT
AMN For	NM_030943.3	TGTCAGTCCTGGTGCAAGAA
AMN Rev		TCTGAGACGCCGAATCCG
Dab2 For	NM_001343.3	GCACCAAAAGCACCCTCAAA
Dab2 Rev		CATCGCCTTTGAACCTTGCT

Table 6. Primer sequences used in Real Time PCR analyses.

Real-time quantitative polymerase chain reaction (qRT-PCR) was performed to quantify messenger RNA levels, using SYBR Green technology. For each fragment to analyze, a reaction mix was made in 20 µl final volume using SYBR Green Master Mix

(EurX) and appropriate primer dilution and annealing temperature as described in Table 7.

Reactions were performed on Rotor-Gene 6000 (Corbett Research, Qiagen) applying the following protocol:

Step 1. 95 °C 10 min

Step 2. 94 °C 15 sec
T_a°C 30 sec
72 °C 30 sec
} 40 cycles

Step 3. From T_a° to 95°C raising by 0.5 °C each step waiting 5 seconds for each step afterwards (Melt Curve)

Data analysis was performed using the ΔΔCt method normalizing on three different housekeeping genes (*GAPDH*, *HPRT1*, *β2-MICROGLOBULIN*) following MIQE guidelines (Bustin et al, 2009).

Name	[PRIMER] µM	T°$_a$ (°C)	Size	Efficiency
GLIC For	0,4	62	92 bp	96%
GLIC Rev	0,4			
B2M For	0,4	62	72 bp	87%
B2M Rev	0,4			
HPRT1 For	0,4	62	140 bp	86%
HPRT1 Rev	0,4			
CLCN5 For	0,2	64	162 bp	99%
CLCN5 Rev	0,2			
CUBN For	0,4	62	118 bp	85%
CUBN Rev	0,4			
LRP2 For	0,4	64	156 bp	98%
LRP2 Rev	0,4			
AMN For	0,1	62	100 bp	96%
AMN Rev	0,4			
Dab2 For	0,4	62	85 bp	97%
Dab2 Rev	0,2			

Table 7. Amplification conditions used in Real Time PCR analyses.

4.10. CUBILIN BLOCKING

To assess whether cubilin mediates albumin uptake, a sheep monoclonal antibody against the extracellular domain of the receptor (R&D Systems) was added to podocyte media. Mature podocytes seeded on 12 well plates were changed in serum free media overnight. Cells were pre-incubated with 6 µg/ml of antibody for 3 h and then stimulated with FITC-BSA 50 µg/ml for 2 hours.

Intracellular signal was measured as previously described in section 4.4. Fluorescence was normalized for the amount of cells measured by methylene blue staining as described elsewhere (Oliver et al, 1989). Briefly cells were stained for 30 min with 1% methyl blue in 0.01 M borate buffer, pH 8.5. After repeated washing with borate buffer, fixed stain, which is proportional to the number of adherent cells, was subsequently eluted with 0.1 N HCl/ethanol 1:1 (vol/vol). Absorbance was measured at 655 nm with the 680 Microplate Reader (Bio-Rad). Results were expressed as fold increase vs control untreated cells. Data are shown as the average ± SD of two different experiments in triplicate.

4.11. MORPHOMETRIC ANALYSIS

Signals for ClC-5, megalin and cubilin on kidney biopsies were quantified by morphometric analysis using Image-Pro Plus 7.0 software (Media Cybernetics). Signals were acquired 200X with the same characteristics of time exposure, gain and intensity among patients. Quantification was performed in both kidney compartment, selecting all glomeruli from each biopsy and the corresponding tubule-interstitium. Quantity was expressed as the mean of area covered by pixels, as a percentage.

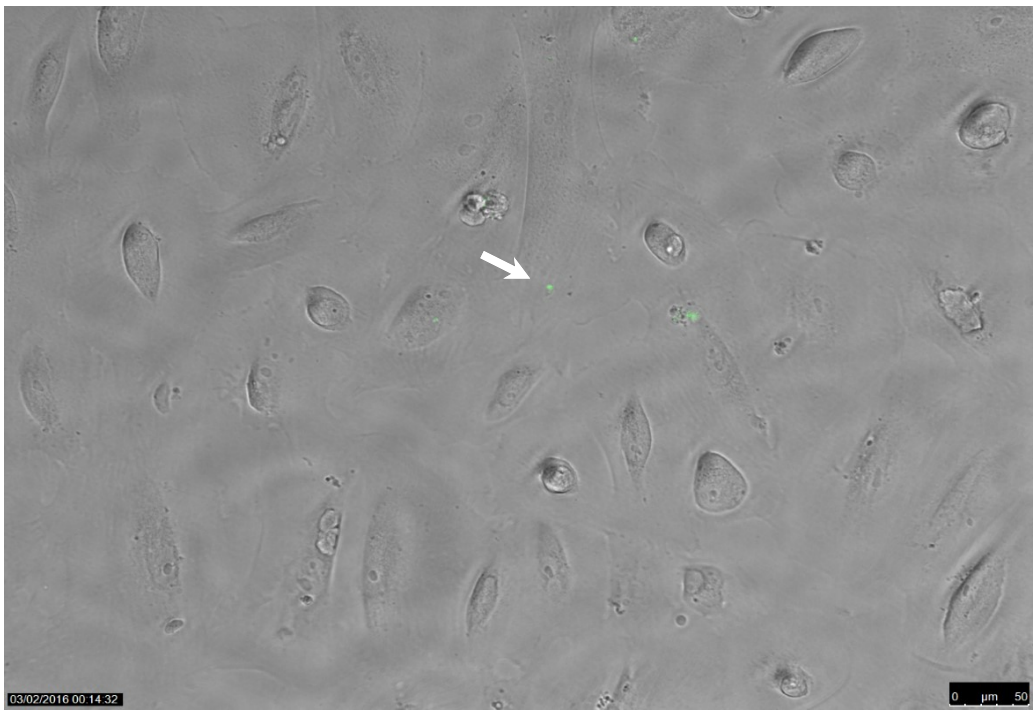
4.12. STATISTICAL ANALYSIS

Statistical analysis was performed by non-parametric tests (Mann-Whitney U test and Spearman's rank correlation). Results with p values below 0.05 were considered as significant. Results were presented as mean ± standard deviation. For Real Time PCR experiments, data were shown as geometrical mean ± standard error.

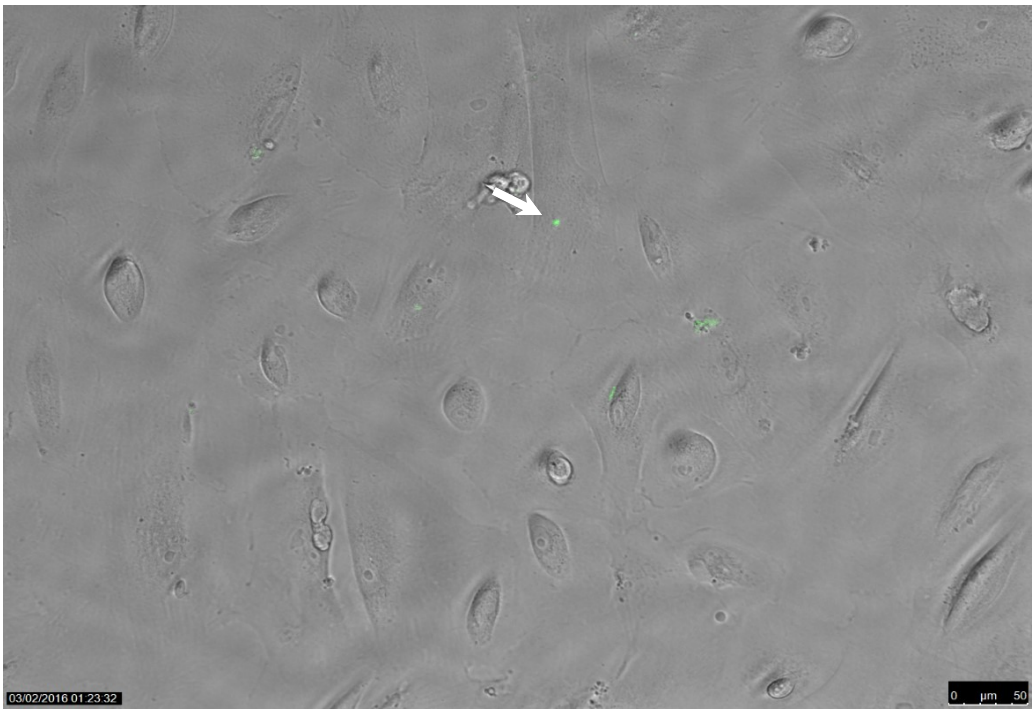
5. Results

5.1. HUMAN PODOCYTES INTERNALIZE ALBUMIN *IN VITRO*

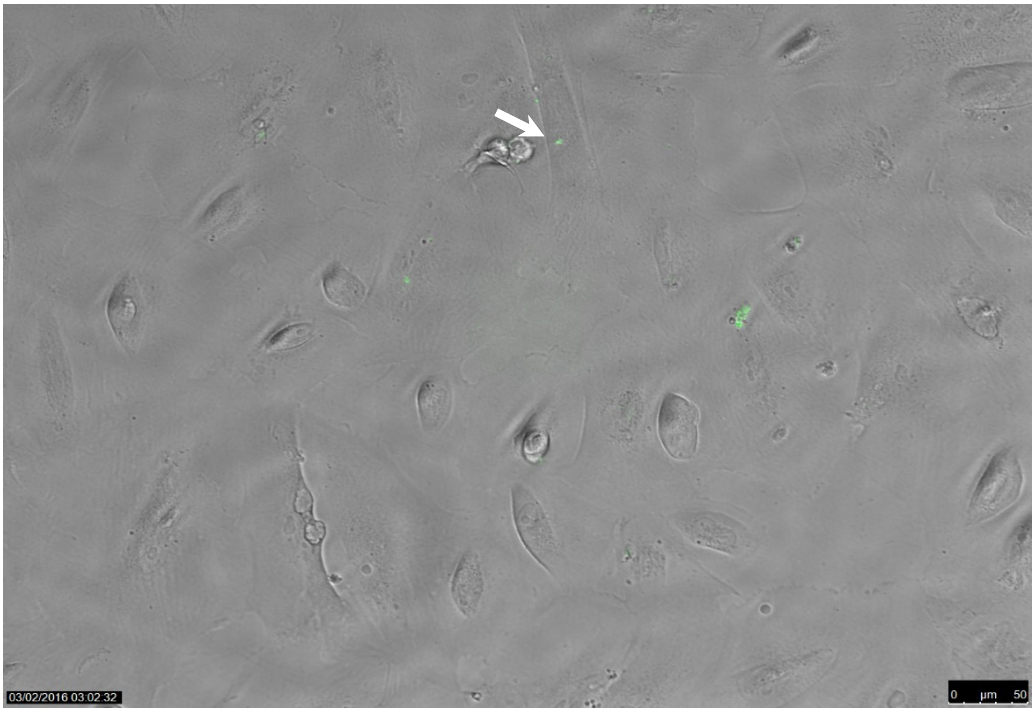
In order to track albumin uptake, we monitored human podocytes overnight after stimulation with a physiological dose of FITC-BSA (10 μg/ml). Starting from 2 hours, we were able to confirm podocyte ability to internalize albumin. The signal was detected as a green vesicle which moved from the cellular membrane to the perinuclear region, finally disappearing (Figure 12). This process, involving 23 ± 9% of the 212 cells observed, started in a range of time between 2 and 15 hours.



A.



B.



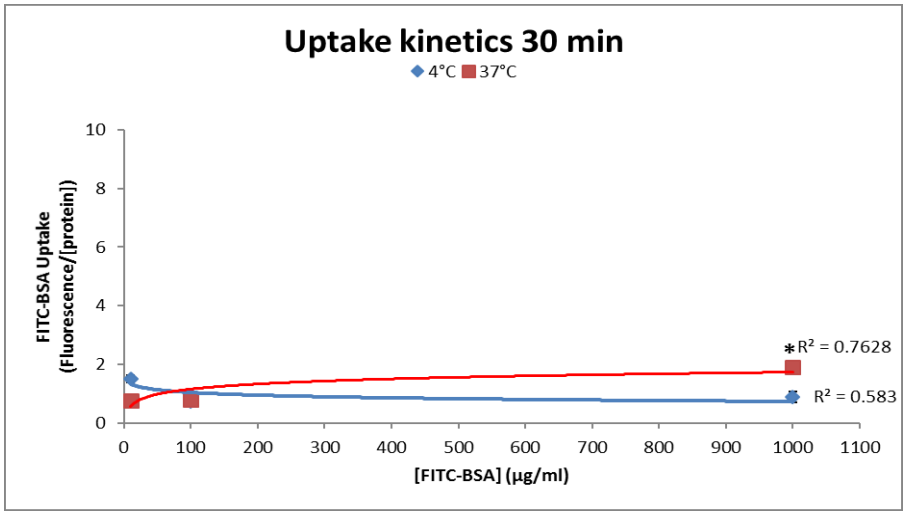
C.

Figure 12. Podocytes internalize albumin. *Representative images of time lapse experiments showing the presence of vesicles containing albumin (green) at different time points (A. 5 h 30 min, B. 6 h 39 min, C. 8 h 18 min). The white arrow indicates the movement from the plasma membrane to the perinuclear region. Magnification: 200X.*

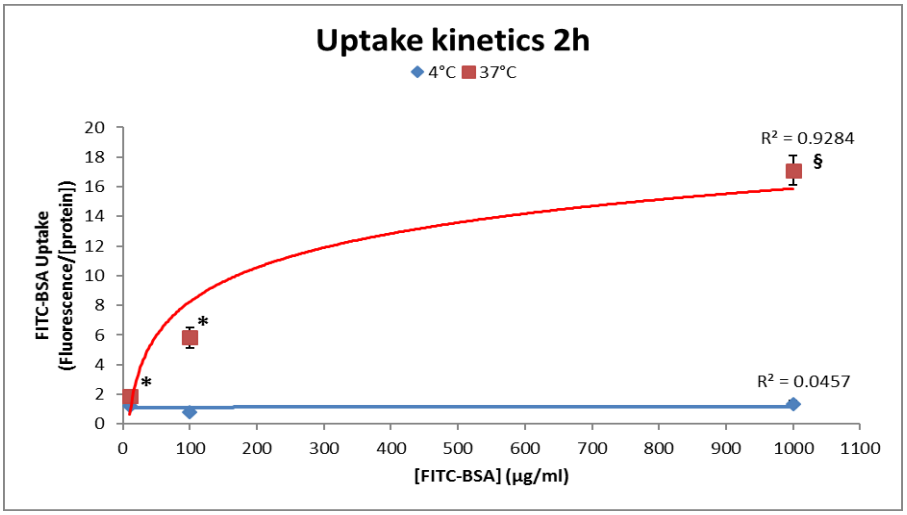
5.2. ALBUMIN ENDOCYTOSIS IN HUMAN PODOCYTES IS A RECEPTOR-MEDIATED PROCESS

In order to establish albumin uptake kinetic parameters in human podocytes, endocytosis and binding experiments were performed. Human podocytes were incubated with increasing concentrations of BSA-FITC at 37 °C (receptor mediated uptake) or 4 °C (non-receptor mediated internalization) and fluorescence intensity was recorded. We observed podocytes after a stimulation of 30 minutes and 2 hours at both temperatures to evaluate the mechanism underling the uptake process.

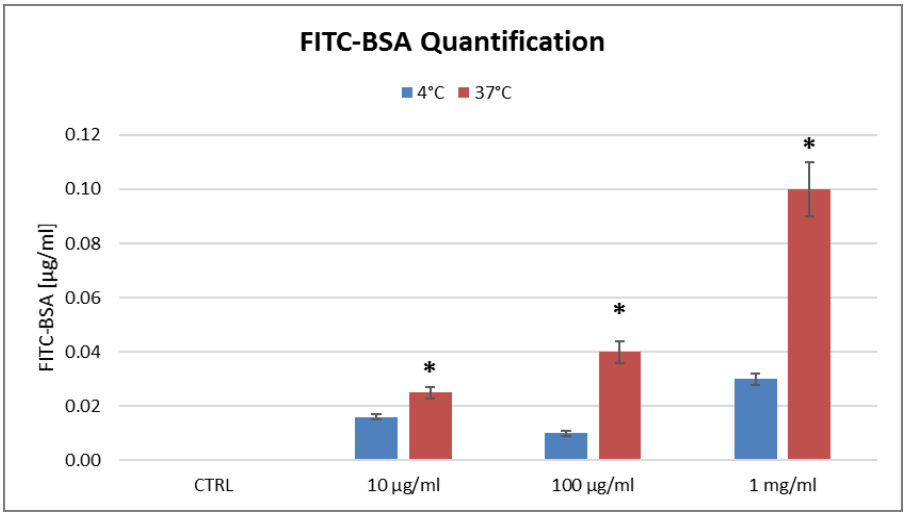
As shown in Figure 13, the uptake process occurred only after 2 hours' stimulation confirming the indication obtained in time lapse experiments. Moreover, the exponential profile obtained at 37 °C but not at 4 °C rule out the non-specific binding of the molecule and support the hypothesis of a receptor-mediated mechanism (Figure 13 B). Finally, FITC-BSA quantification indicated that the uptake process at 37 °C followed a significant dose dependent increase (10 µg/ml: 16.99 ± 3.06 µg albumin/mg protein, 100 µg/ml: 26.31 ± 7.18 µg albumin/mg protein, and 1 mg/ml: 53.31 ± 10.68 µg albumin/mg protein) comparing to 4 °C (Figure 13 C).



A.



B.



C.

Figure 13. Albumin uptake follows a receptor-mediated kinetic in human podocytes. *At 30 minutes only the 1 mg/ml displayed significance (A.) while after 2 hours, FITC-BSA was significantly internalized at all doses tested (B.) comparing to control cells. Albumin uptake increased until saturation at 37 °C (red) with abolition of the process at 4 °C (blue). Graphic C. displays significant FITC-BSA quantity internalized by podocytes at 37 °C comparing to 4 °C. Data are shown as the average ± SD of three different experiments in triplicate. * p<0.05; § p<0.01.*

5.3. HUMAN PODOCYTES EXPRESS TUBULAR PROTEIN UPTAKE MACHINERY COMPONENTS

Since our results indicated the presence of a receptor-mediated mechanism involved in albumin internalization, we evaluated *in vitro* the presence of this system in mature podocytes displaying podocin positivity (Figure 14 C.).

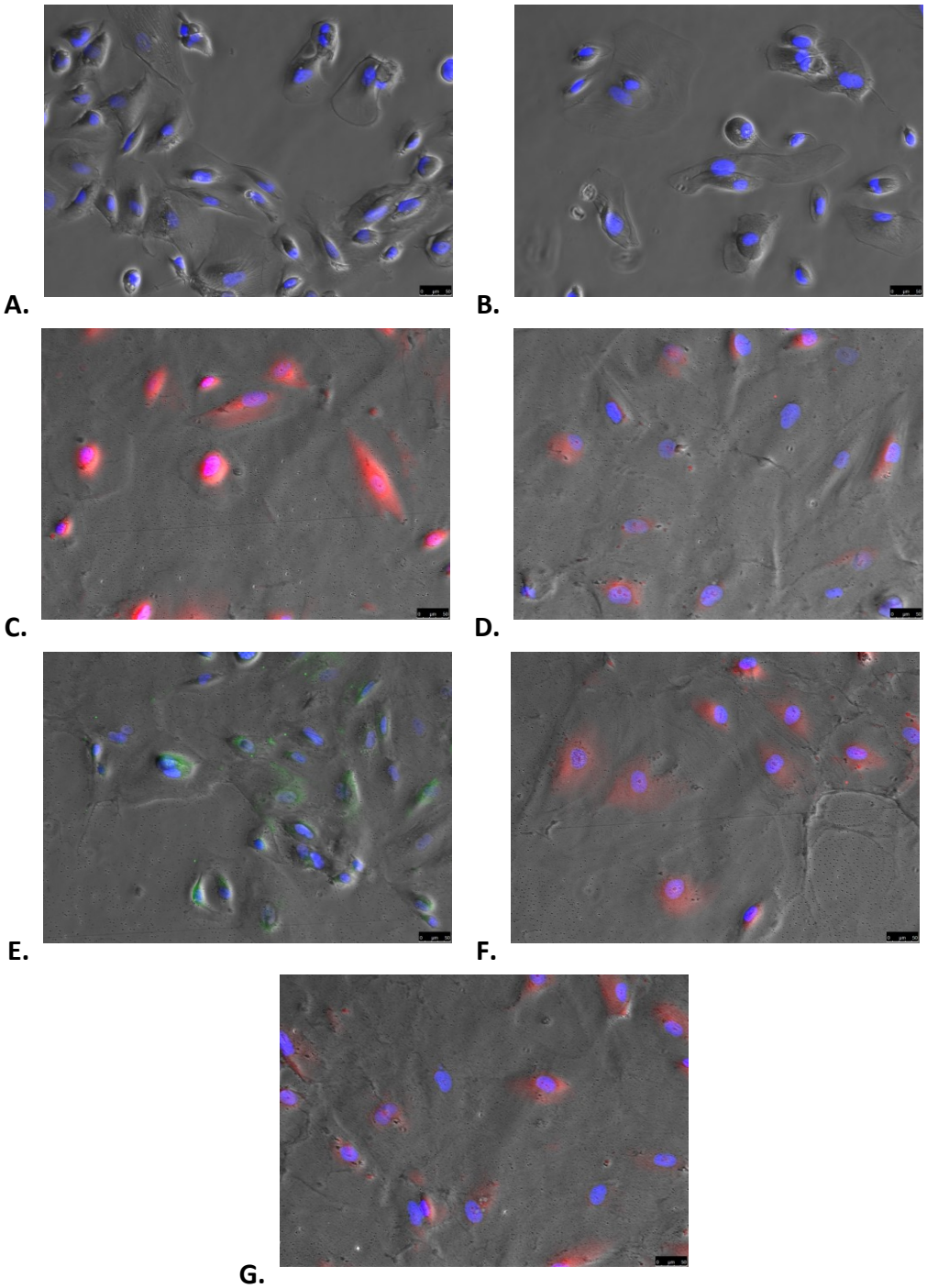
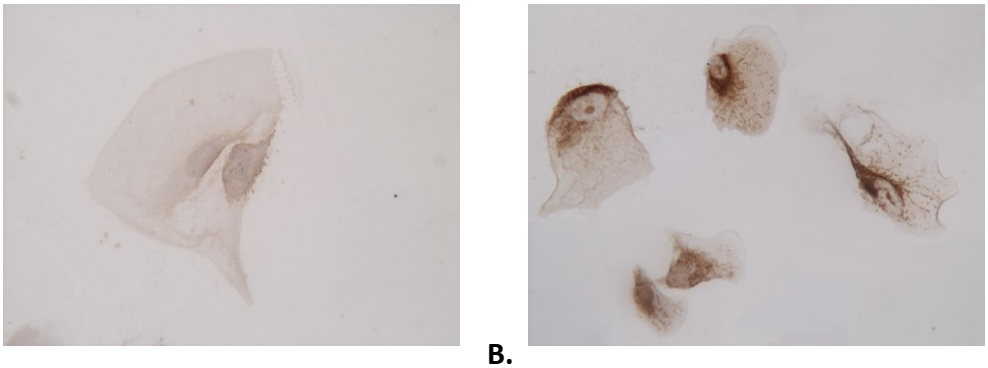


Figure 14. Human mature podocytes express the tubular uptake machinery. *Representative images showing the presence of: C. Podocin (red), D. megalin (red), E. cubilin (green), F. Dab2 (red), G. AMN (red). A. rabbit and B. sheep negative controls. Blue: DAPI. Magnification 200X.*

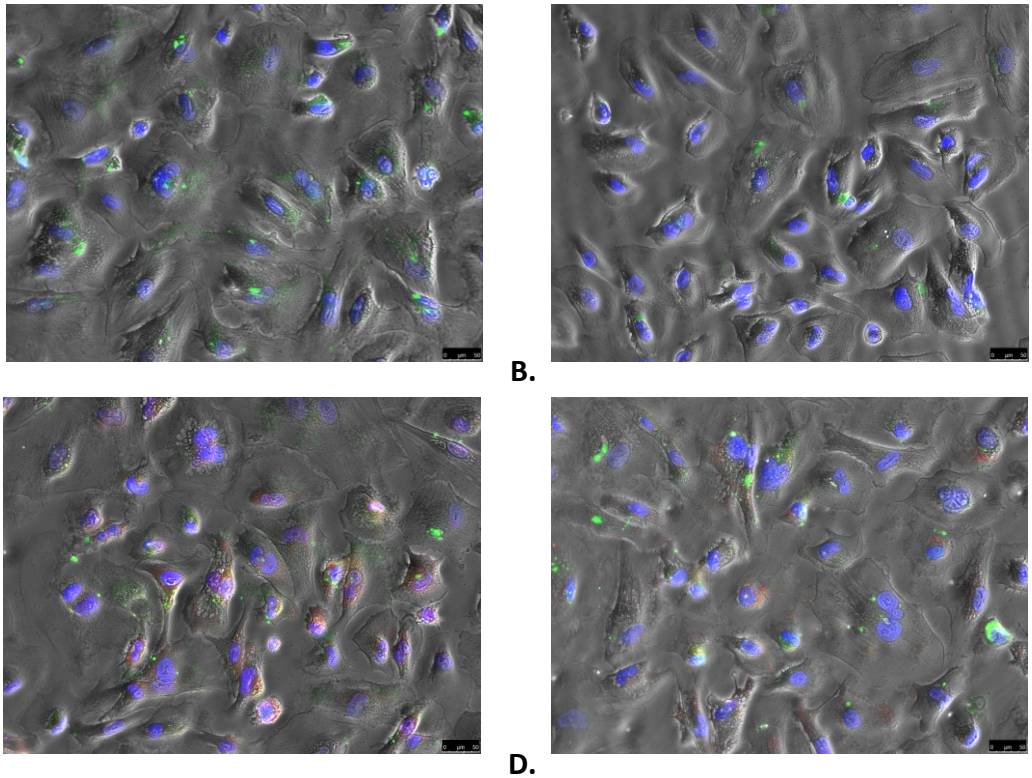


A. B.

Figure 15. Human mature podocytes express ClC-5. *Representative images showing the presence of ClC-5. A. Negative control, B. ClC-5 (brown). Magnification 200X.*

As reported in Figure 14 and Figure 15 we confirmed the presence of megalin and cubilin, and for the first time we highlighted the presence of Dab2, AMN and ClC-5 in human mature podocytes *in vitro*.

5.4. MEGALIN AND CUBILIN CO-LOCALIZE WITH ALBUMIN VESICLES



A. B. C. D.

Figure 16. Megalin and cubilin co-localize with albumin. *Representative images demonstrating the co-localization (in yellow/orange) between megalin (C.) and cubilin (D.) with albumin after 24 hours' incubation. A. rabbit negative control, B. sheep negative control. Green: FITC-BSA; Red: megalin (C.) or cubilin (D.); Blue: DAPI. Magnification 200X.*

Since albumin can be internalized *via* megalin or cubilin, or both, we investigated whether these receptors were involved in albumin binding. After 2, 4, 8 and 24 hours' incubation with FITC-BSA 10 µg/ml, mature podocytes were immunostained for the evaluation of megalin and cubilin localization.

The highest co-localization signal was more evident after 24 hours' stimulation as shown in Figure 16 C and D. Moreover, the signal was mainly perinuclear confirming the movement of BSA vesicles from the membrane to the nucleus observed in time lapse experiments.

5.5. ALBUMIN MODULATES PROTEIN UPTAKE SYSTEM EXPRESSION *IN VITRO*

Since we identified the presence of the components of the protein uptake machinery in mature human podocytes, we wanted to investigate whether protein overload could affect the expression level of this system.

In order to examine whether albumin stimulation induced deleterious effects on human podocytes, we first evaluated the percentage of dead cells at 24, 48 and 72 hours after incubation with BSA (range 10 µg/ml - 30 mg/ml). We disclosed no significant increase in necrosis and/or apoptotic events at all time and concentrations tested (Figure 17).

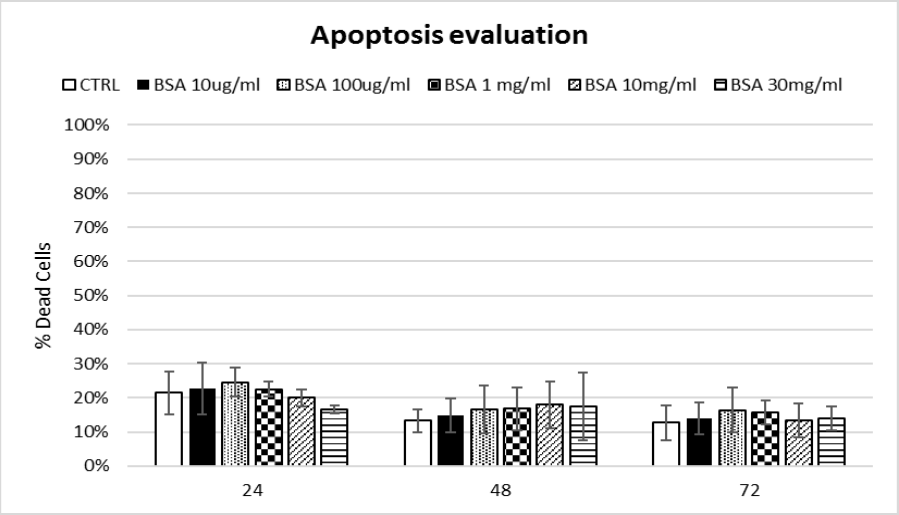
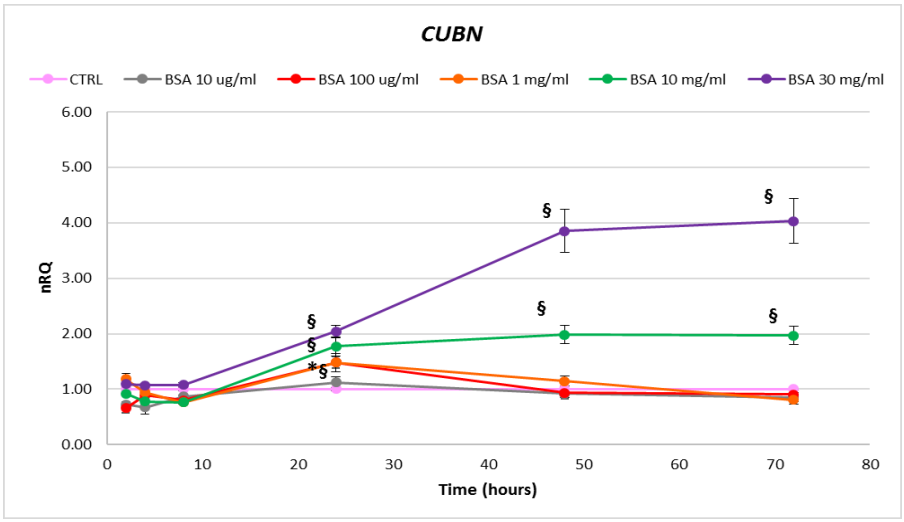


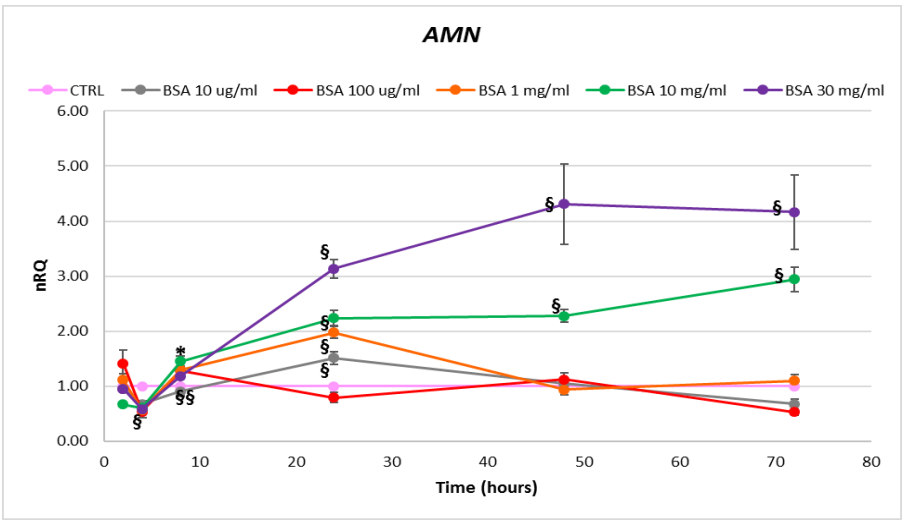
Figure 17. Albumin did not affect cell death. *Evaluation of cell death after albumin stimulation at different time and doses. No significant differences were observed comparing to control cells.*

Gene expression data indicated a significant increase in *CUBN* and *AMN* expression especially at higher BSA concentration (10 and 30 mg/ml) and in longer time (24, 48 and 72 hours) (Figure 18 A. and B.). Also *CLCN5* expression was significantly affected

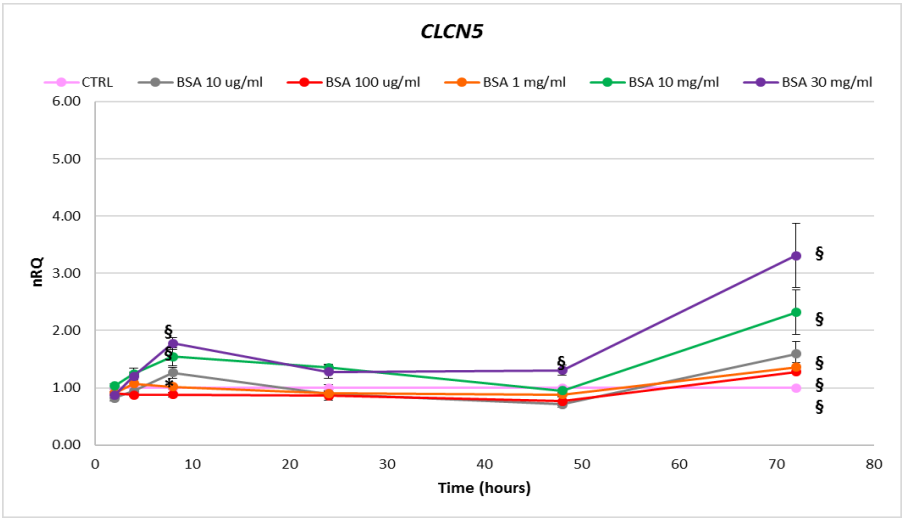
by chronic stimulation (72 hours) at all concentration tested (Figure 18 C.) while *DAB2* was upregulated at all concentration tested only at 24 hours (Figure 18 D.). BSA stimulation didn't affect *LRP2* expression, which was expressed at very low levels.



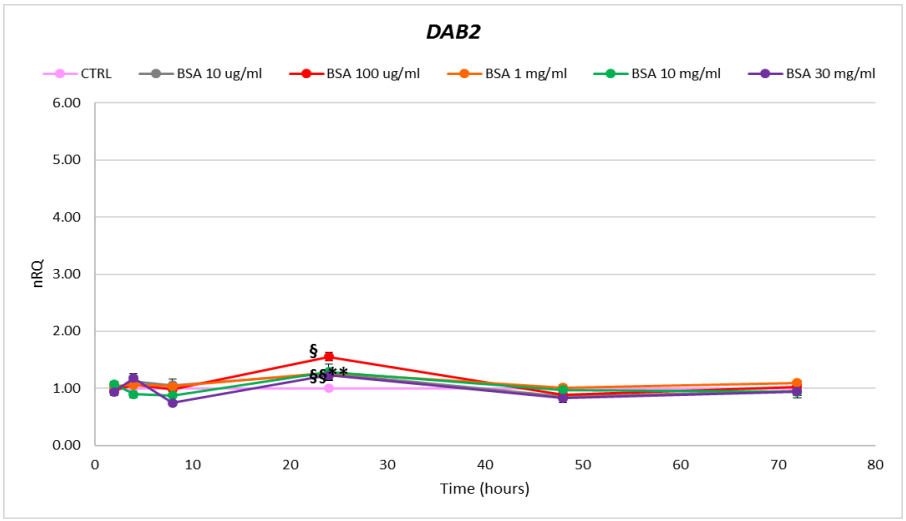
A.



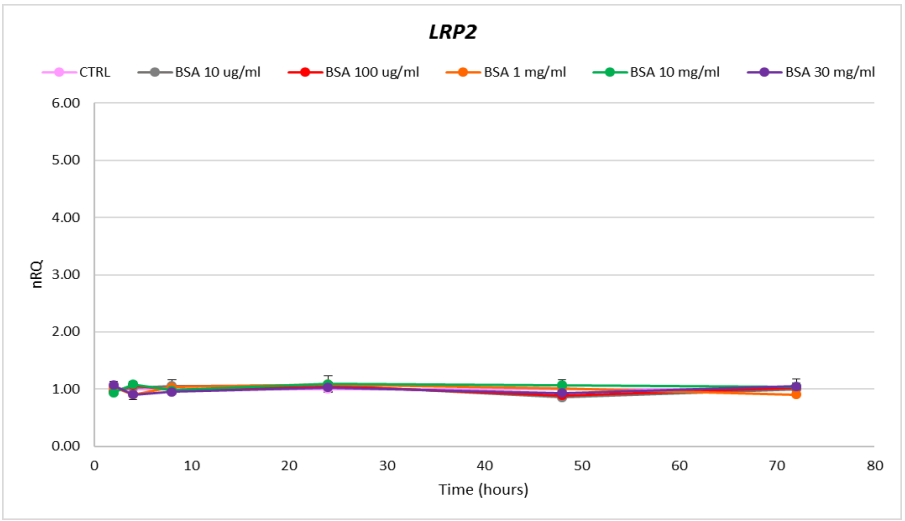
B.



C.



D.



E.

Figure 18. Albumin modulates protein uptake machinery expression in human podocytes. *Expression analysis of the macromolecular system displayed an increase in* CUBN *(A),* AMN *(B),* CLCN5 *(C) and* DAB2 *(D). No effects were observed on* LRP2 *expression (E.). Data are shown as the geometrical average ± SE of three experiments in duplicate. nRQ: normalized Relative Quantity. CTRL: control cells. *p<0.05, §p<0.01.*

5.6. CUBILIN MEDIATES ALBUMIN ENDOCYTOSIS IN HUMAN PODOCYTES

Since gene expression analysis indicated a possible involvement of the CUBAM complex, we wanted to confirm this data at functional level. We inhibited albumin binding sites on cubilin and quantified internalized FITC-BSA after 2 hours' stimulation. We used BSA concentration of 50 µg/ml in order to position the experiment in the exponential phase of the uptake kinetic.

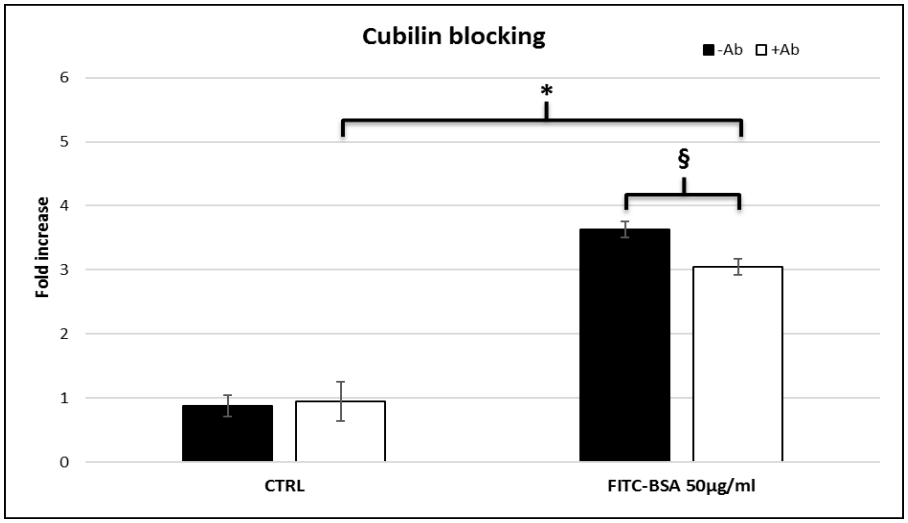


Figure 19. Cubilin mediates albumin endocytosis. *Antibody-mediated inhibition of cubilin (white blocks) leaded to a significant decrease in albumin endocytosis indicating an involvement of this receptor in the protein uptake process in mature podocytes. Data are shown as the average ± SD of two different experiments in triplicate. CTRL: control cells. * p<0.05, § p<0.01.*

As shown in Figure 19 we observed a significant reduction of albumin internalization, indicating that in human podocytes the CUBAM complex is involved in this process. However, although a significant inhibitory effect was observed, albumin internalization still happens indicating that, when cubilin pathway is blocked, albumin endocytosis may occur through alternative pathways. These results suggest that the cubilin-facilitated path is not the exclusive endocytic pathway for albumin in human podocytes.

5.7. ClC-5, MEGALIN, AND CUBILIN ARE EXPRESSED AT GLOMERULAR LEVEL IN PROTEINURIC PATIENTS

We evaluated the presence and the expression levels of ClC-5 by IHC, and of megalin and cubilin by IF in serial sections of 6 control biopsies, 23 SLE patients and one case of MCD (as example of glomerulonephritis caused by injury of non-immunological origin). We observed the presence of ClC-5, megalin and cubilin both at tubular and glomerular level in both control biopsies and proteinuric patients (Figure 20 and 21).

Moreover, morphometric analysis disclosed an unsurprising significant higher signal at tubular than glomerular level for all the protein analyzed in both controls and SLE patients (Figure 22). *In vivo* data are supported by previous *in vitro* experiments, since we observed the presence these components of the macromolecular system the glomerular compartment.

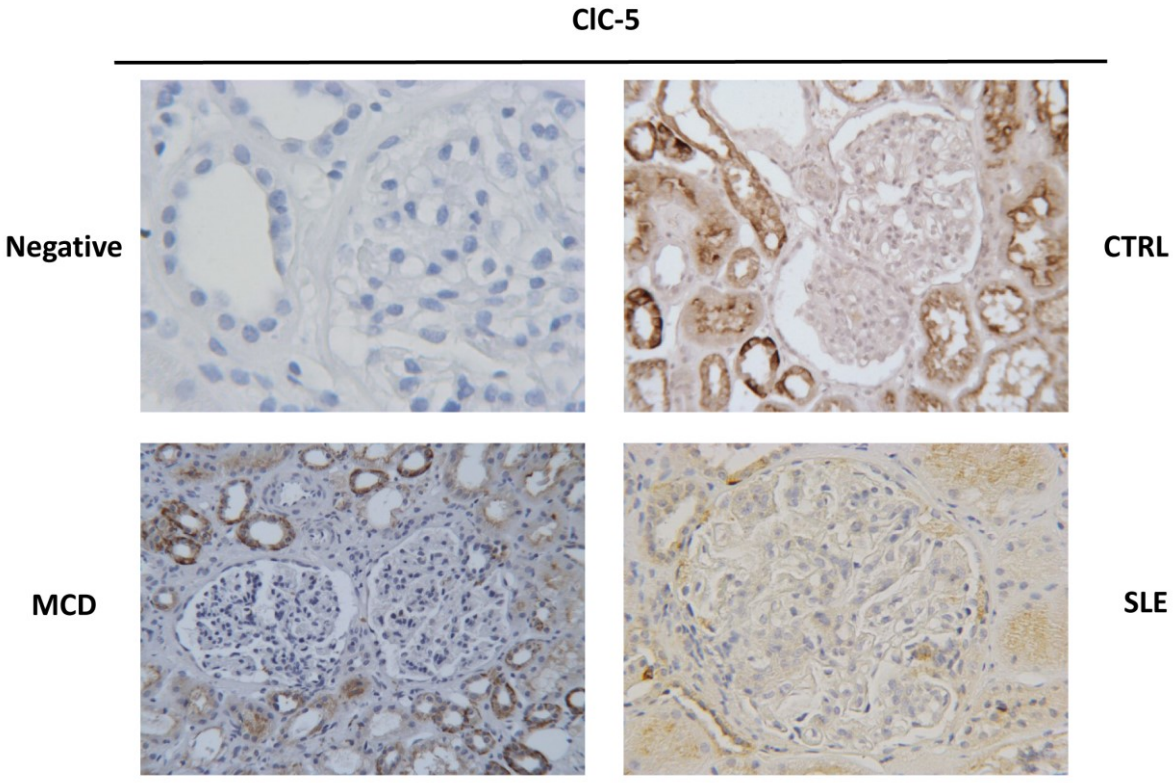


Figure 20. ClC-5 is expressed in tubules and glomeruli of controls and proteinuric patients.
Representative images displaying ClC-5 positivity. Brown: ClC-5. Negative: omitting primary antibody, CTRL: controls, MCD: Minimal Change Disease, SLE: Systemic Lupus Erythematous. Magnification 200X.

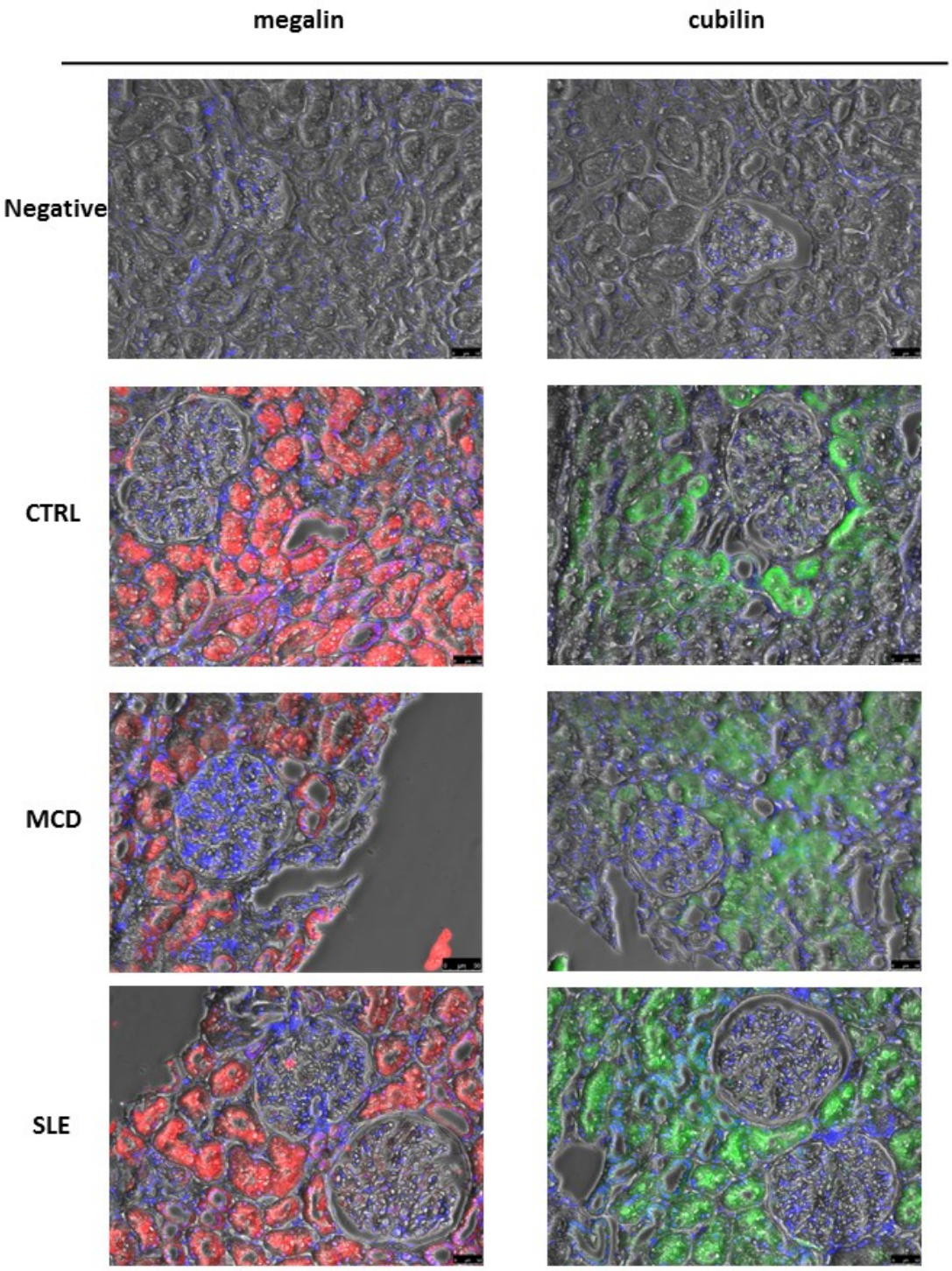
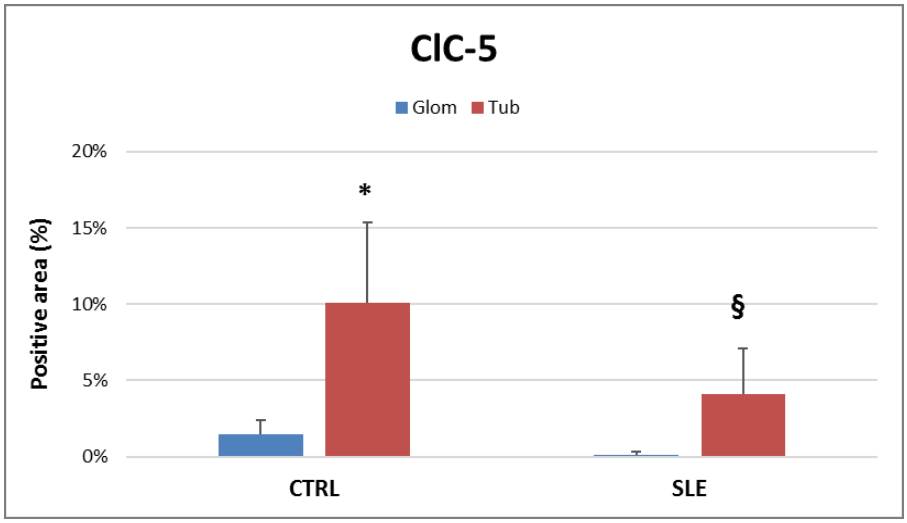
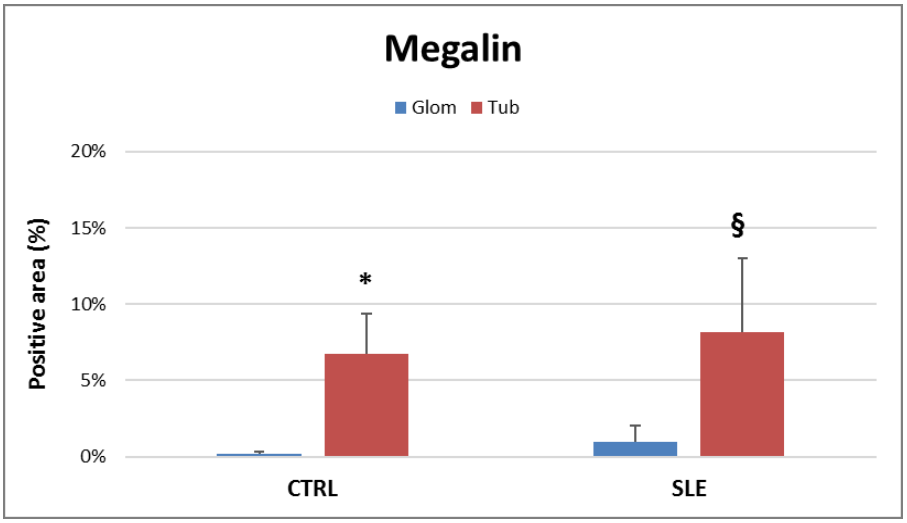


Figure 21. Megalin and cubilin are expressed in tubules and glomeruli of controls and proteinuric patients. *Representative images displaying megalin and cubilin positivity. Red: megalin, Green: cubilin, Blue: DAPI. Negative: omitting primary antibody, CTRL: controls, MCD: Minimal Change Disease, SLE: Systemic Lupus Erythematous. Magnification 200X.*



A.



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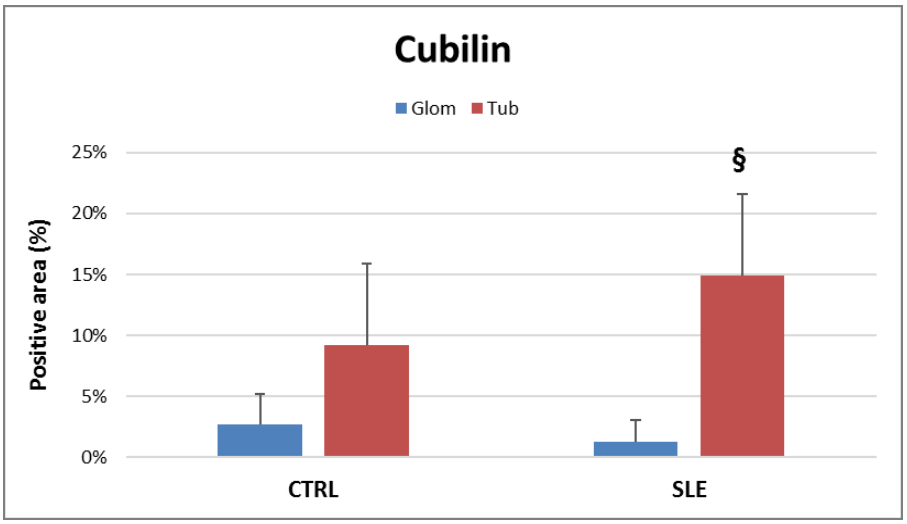


C.

Figure 22. Protein expression analysis between controls and SLE patients. *We observed higher expression level of the three molecules investigated at tubular vs glomerular level both in controls (CTRL) and SLE patients. A. ClC-5, B. megalin, C. cubilin. Data are shown as the average ± SD. * p<0.05, § p<0.01. Glom: Glomerular expression, Tub: Tubular expression.*

Comparing system protein expression between controls and SLE patients, we did not observe a significant difference in protein levels in both compartments (Figure 23).

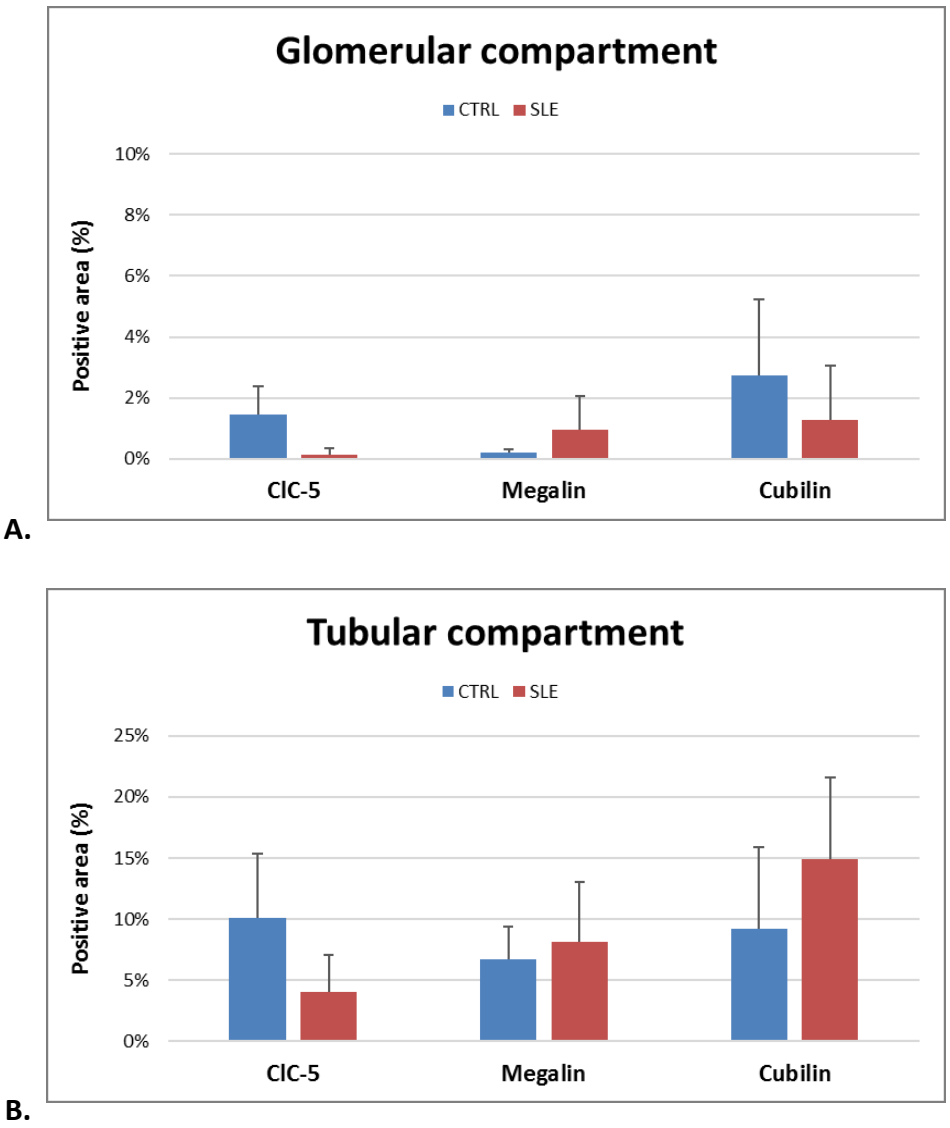
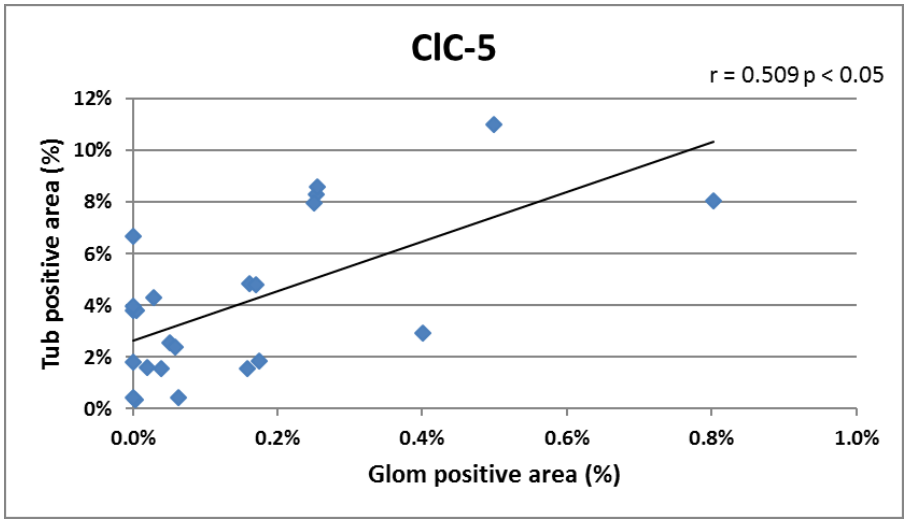


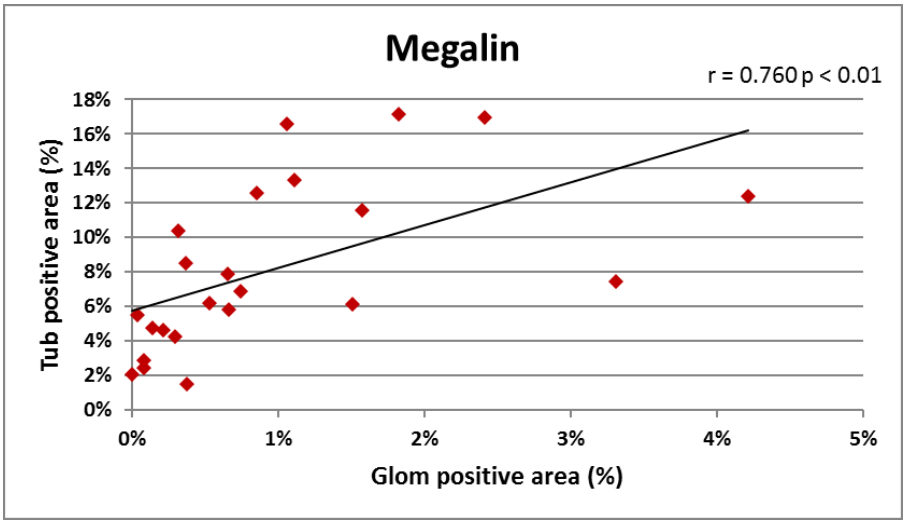
Figure 23. Protein expression analysis between controls and SLE patients. *We observed no differences in the expression of ClC-5, megalin and cubilin between control and SLE biopsies in both compartments. A. glomerular expression, B. tubular expression. Data are shown as the average ± SD. CTRL: control biopsies, SLE: Systemic Lupus Erythematous biopsies.*

Intriguingly, statistical analysis revealed a positive correlation between the two compartments in the expression of ClC-5, megalin and cubilin (Figure 24) in SLE patients.

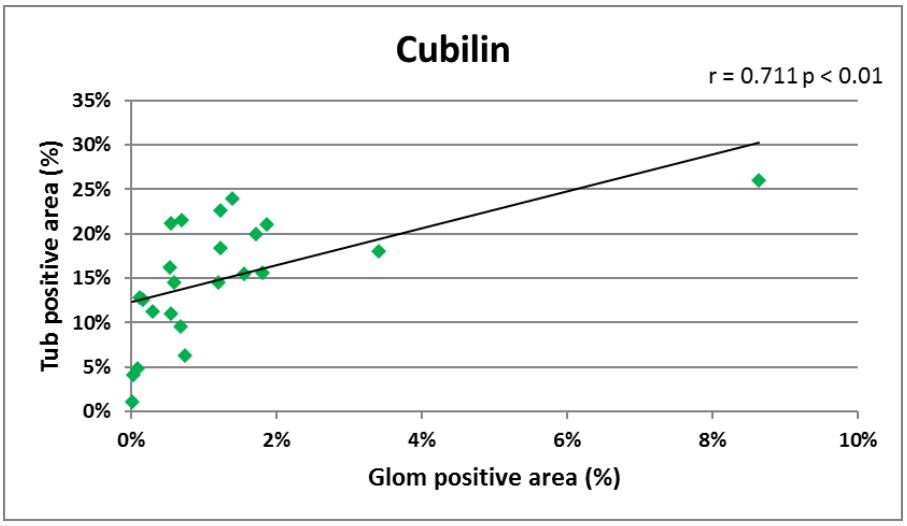
Same analysis could not be performed on control biopsies due to the small cohort of patients.



A.



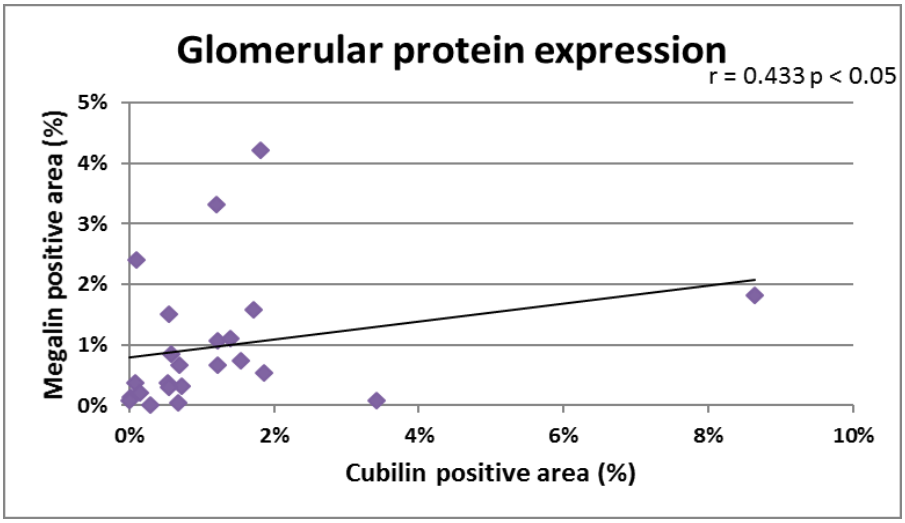
B.



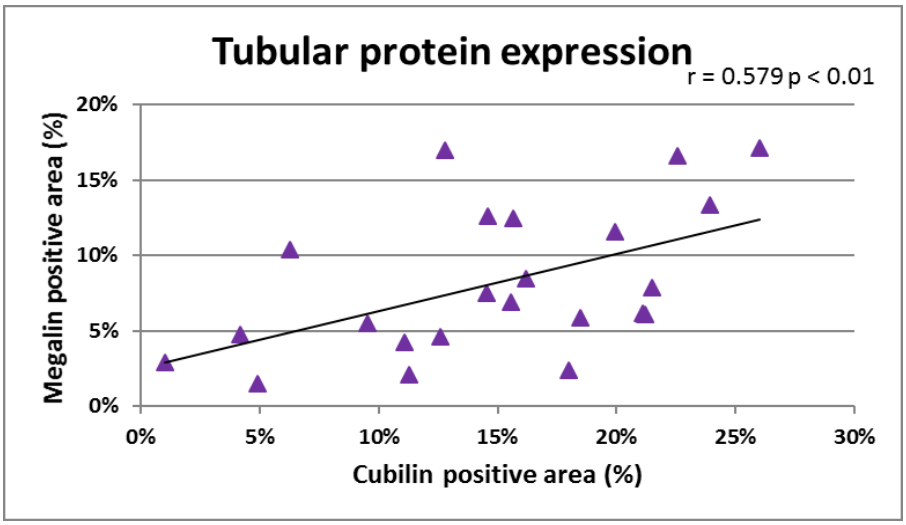
C.

Figure 24. Correlation analysis on SLE patients. *We disclosed a significant linear correlation in the expression level between tubular (Tub) and glomerular (Glom) ClC-5 (A.), tubular and glomerular megalin (B.), and tubular and glomerular cubilin (C.).*

Furthermore, we observed a significant correlation between megalin and cubilin protein expression in both compartments (Figure 25), while no significant correlation was observed considering ClC-5 vs both receptors.



A.

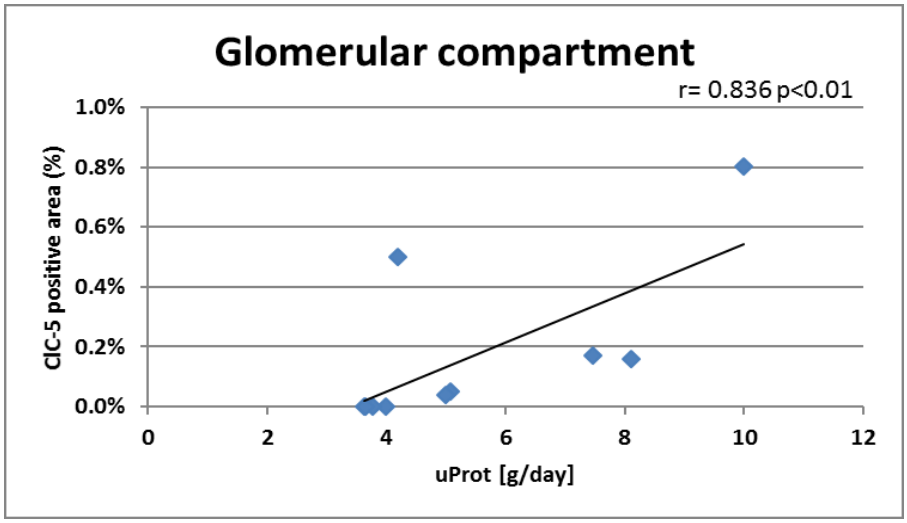


B.

Figure 25. Correlation between megalin and cubilin on SLE patients. *We disclosed a significant linear correlation between megalin and cubilin expression level in the tubular and glomerular compartments. A. Glomerular signal, B. Tubular signal.*

In order to evaluate the possible role of proteinuria on the modulation of the endocytic macromolecular complex expression, we compared patients presenting nephrotic syndrome (n= 10, range 3.66 - 10 g/day) vs patients with low level of proteinuria (n= 13, range 0.56 - 3 g/day). We disclosed positive correlations between ClC-5 and megalin in the glomerular compartment vs uProt and between tubular megalin vs uProt only in the nephrotic patients group (Figure 26).

A.



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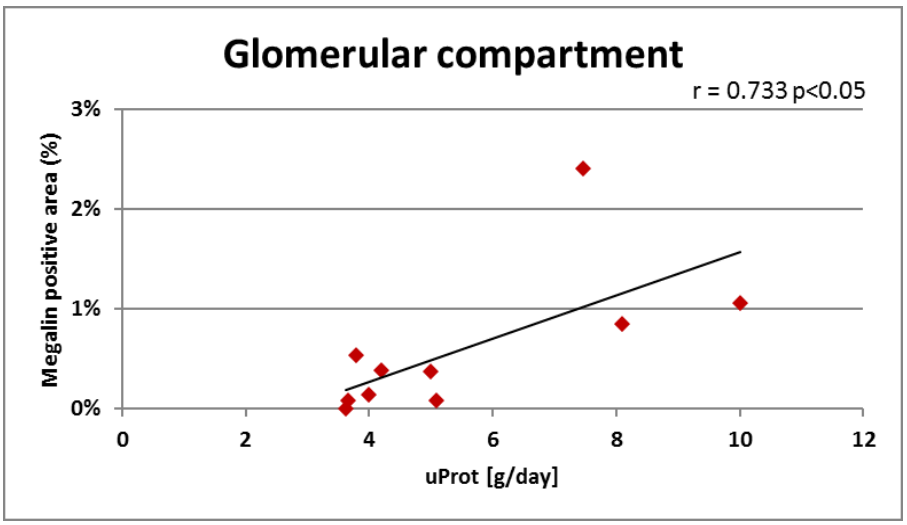


C.

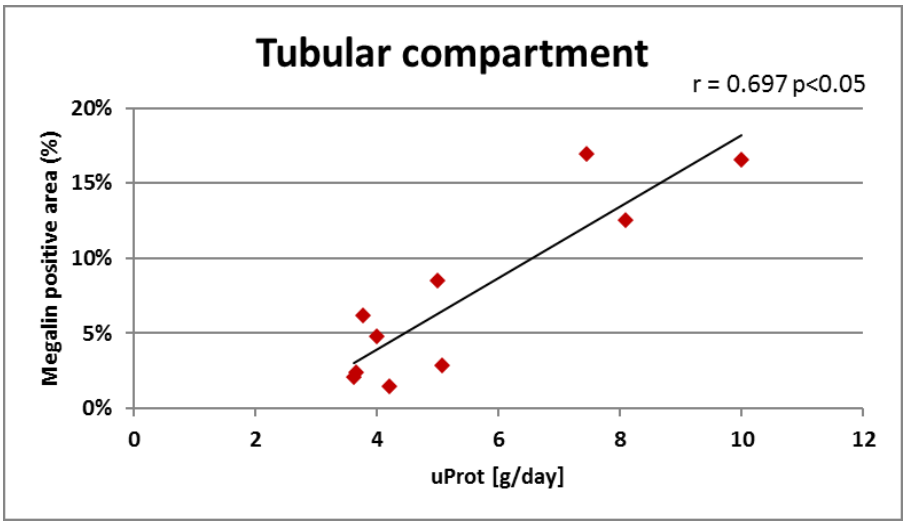


Figure 26. Correlation with proteinuria in nephrotic SLE patients. *We disclosed a positive correlation in patients with nephrotic syndrome between proteinuria and ClC-5 glomerular expression and between proteinuria and both tubular and glomerular expression of megalin. A. glomerular ClC-5, B. glomerular megalin, C. tubular megalin.*

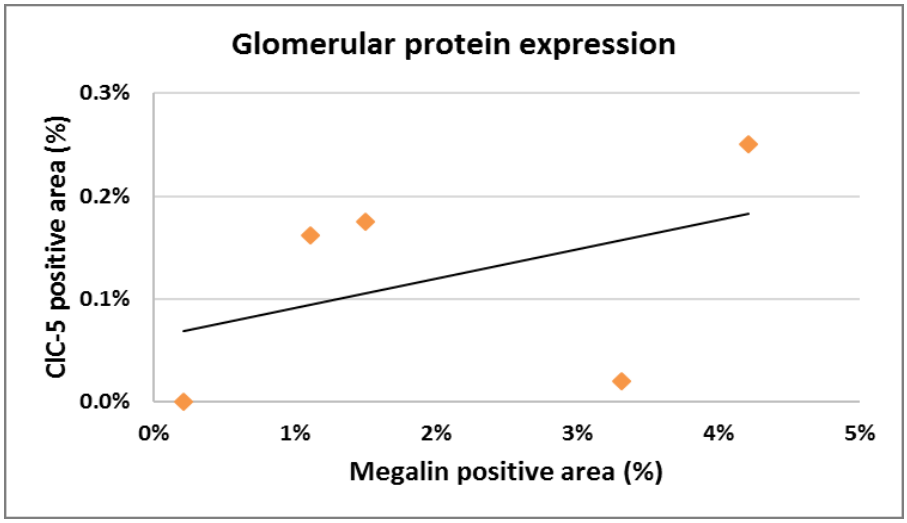
In order to better understand the influence of the pharmacological therapy on the expression of ClC-5, megalin and cubilin, we divided patients in two groups: treated (n= 13) or not treated (n= 10) with ACEi or ARB. We found no significant differences between the two groups in the expression pattern of this system.

Moreover, we found no significant differences also dividing patients between treated (n= 14) or not treated (n= 9) with immunosuppressive drugs.

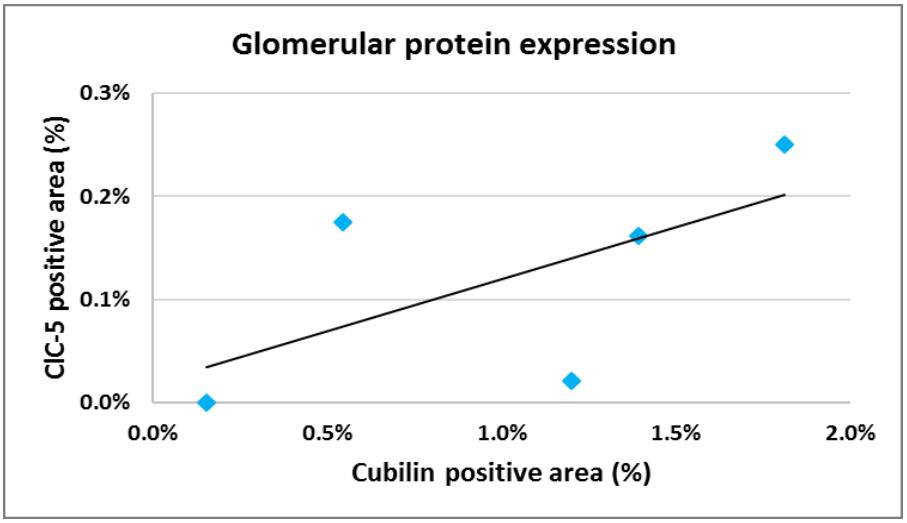
Interestingly, we disclosed a positive trend between ClC-5, megalin and cubilin at glomerular level selecting only patients without both ACEi/ARB and immunosuppressive drugs (n= 5) ($r= 0.7$ $p> 0.05$) (Figure 27).

Considering the histological classification, we observed higher level of tubular megalin in Class IV-V/V (n= 5) vs Class II/III patients (n= 5) ($p< 0.05$) (Figure 28).

No other significances were observed.



A.



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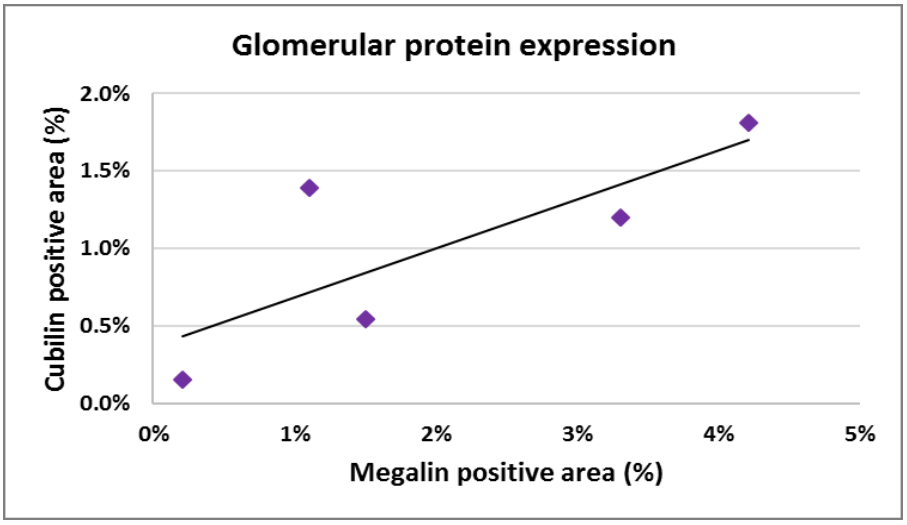
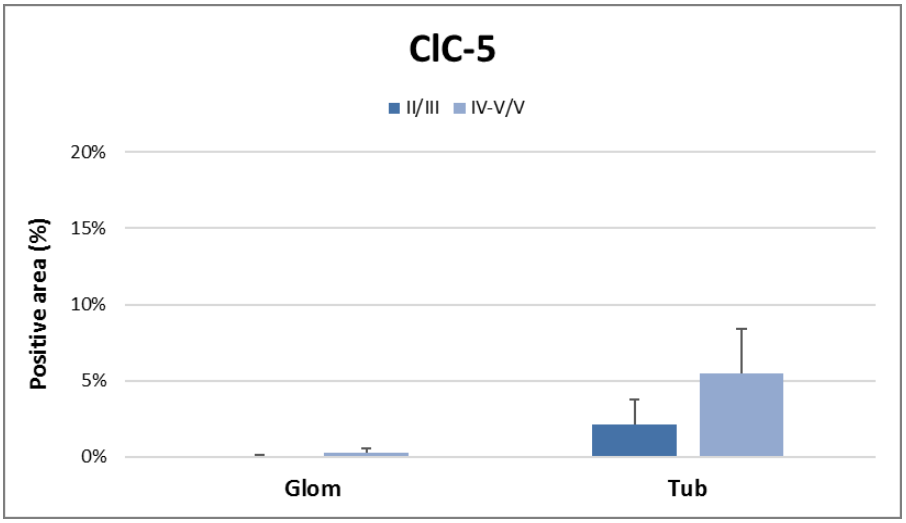
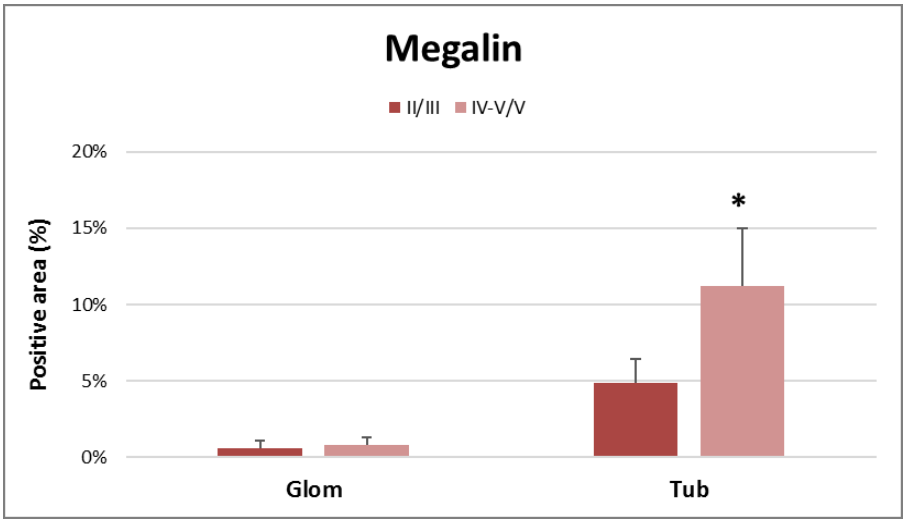


C.

Figure 27. Correlation between ClC-5, megalin and cubilin on SLE patients without pharmacological treatment. *We disclosed positive trend between the expression level of the three proteins in the glomerular compartments. A. ClC-5 vs megalin, B. ClC-5 vs cubilin, C. megalin vs cubilin.*



A.



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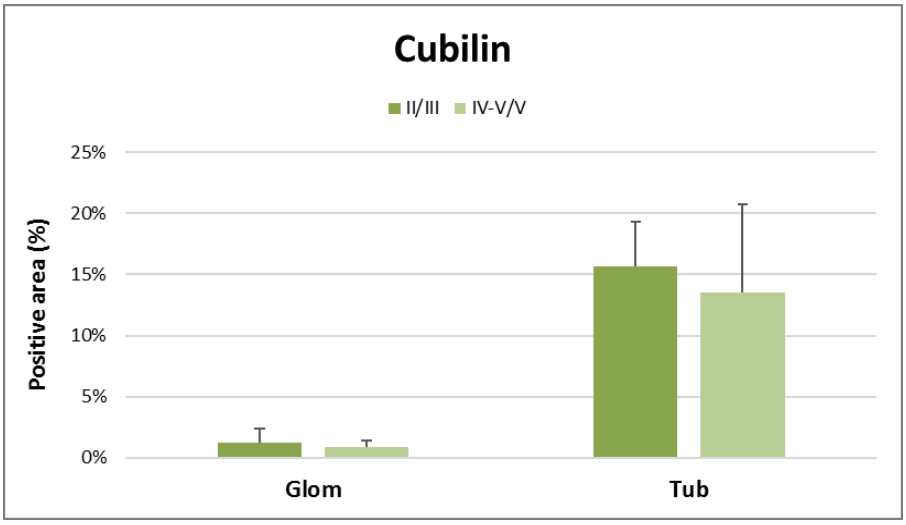

C.

Figure 28. Evaluation of protein expression dividing SLE patients by histological classification. *We disclosed significant higher level of tubular megalin in later stages of SLE. A. ClC-5, B. megalin, C. cubilin. * p<0.05.*

5.8. MEGALIN, CUBILIN AND CLC-5 ARE EXPRESSED IN PECS OF SLE PATIENTS

Analyzing SLE patients we observed a fascinating extended positivity pattern localized presumptively in parietal epithelial cells in about 30 % of the patients. The staining was detected in about 10 - 15 % of the analyzed glomeruli. This pattern was not present in controls and in the only case of MCD analyzed (Figure 29).

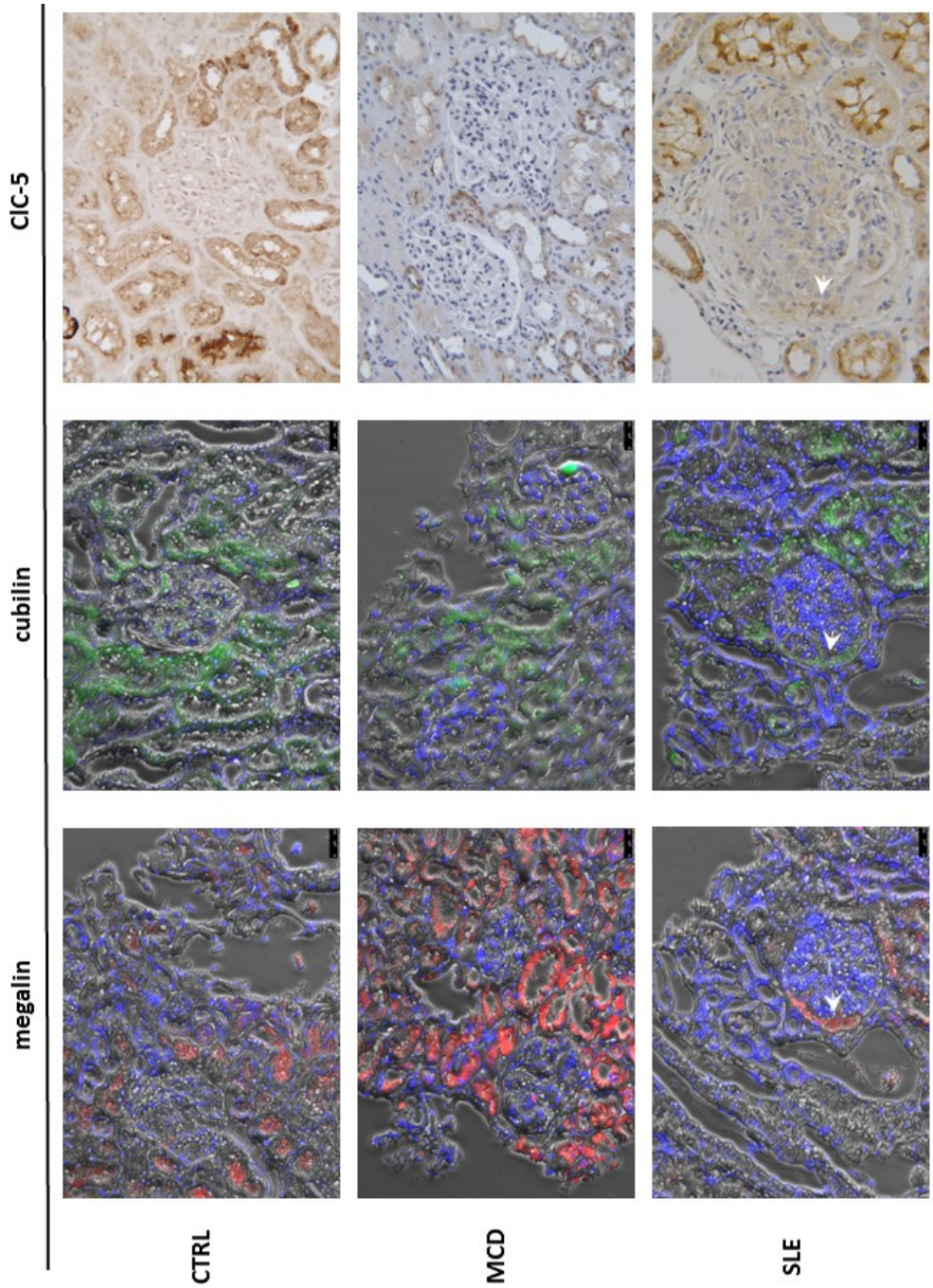

Figure 29. Glomerular positivity in parietal cells. Representative images *displaying an extensive positivity (white arrows) for megalin (A), cubilin (B) and ClC-5 (C) at parietal level in SLE patients but not in controls (CTRL) and MCD. Blue: DAPI, Red: megalin, Green: cubilin, Brown: ClC-5. Magnification 200X.*

In order to confirm that these cells express both receptors we performed double immunofluorescence staining. As shown in Figure 30, megalin and cubilin signals co-localize in these hypertrophic cells.

In serial sections of kidney biopsies of SLE patients presenting this unusual pattern, we demonstrated that the region of interest was ANXA3 (mature) and CD24 (progenitors) positive, but CD44 (activated) negative (Figure 31).

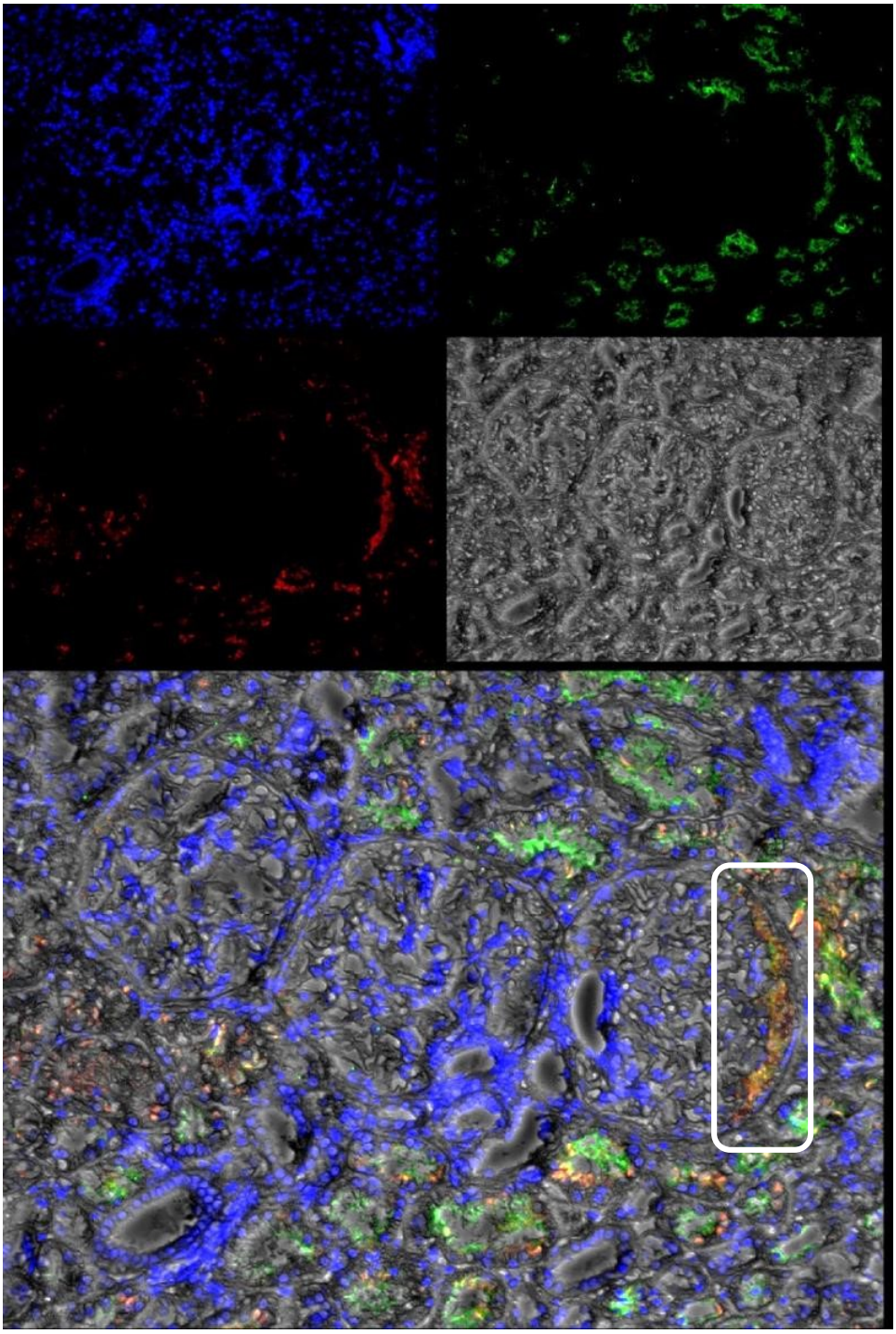

Figure 30. Megalin and cubilin co-localize at parietal level of SLE patients. Representative images *displaying the double staining for cubilin and megalin (white rectangle). Green: cubilin, Red: megalin, Blue: DAPI, Orange/Yellow: co-localization between cubilin and megalin. Magnification 200X.*

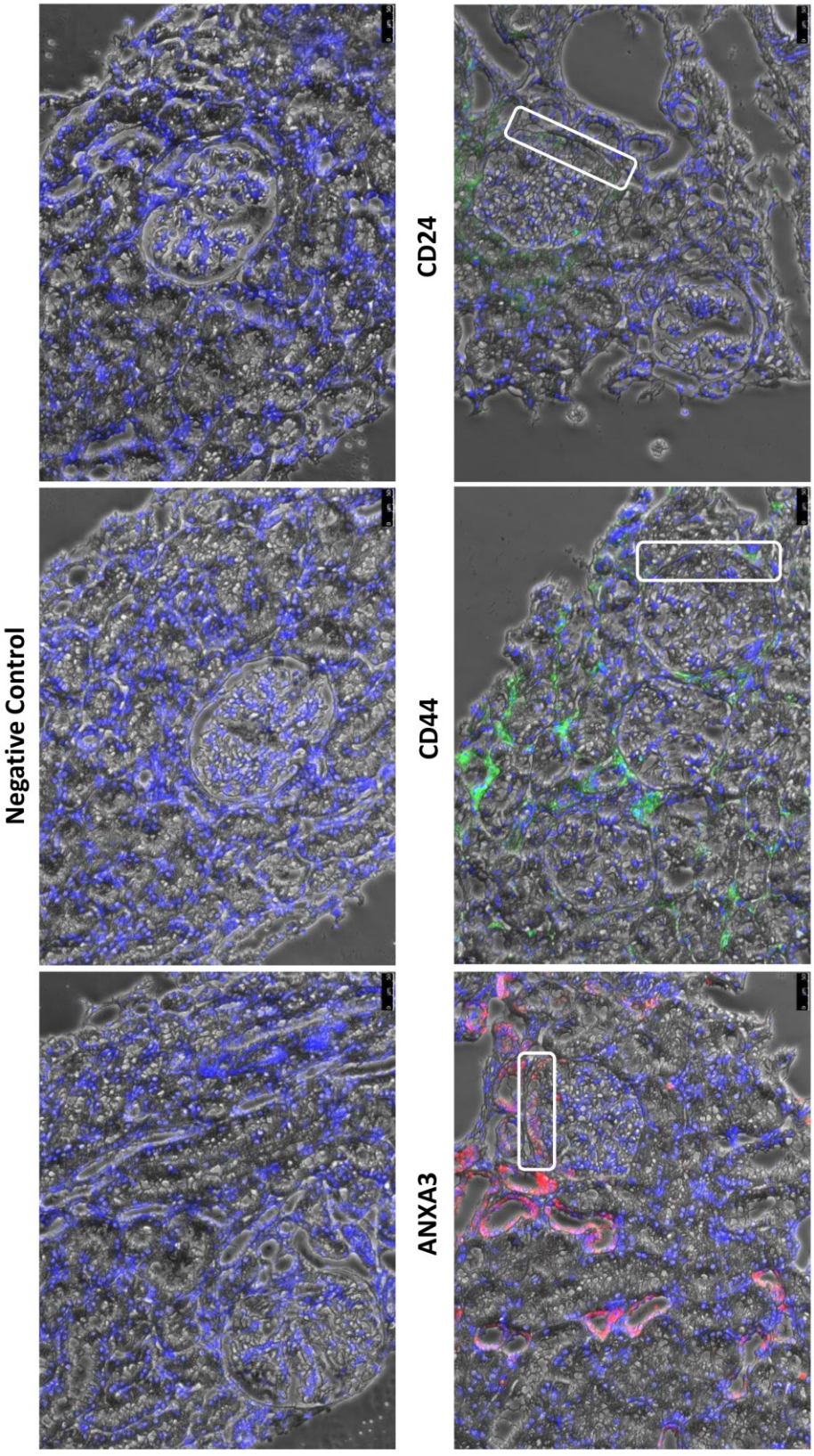

Figure 31. Characterization of the nature of megalin and cubilin positive PECs in SLE patients. *Immunofluorescence showing the expression pattern of the region previously identified as megalin^{+}/cubilin^{+} (surrounded by white rectangle). Blue: DAPI, Red: ANXA3, Green CD44 or CD24 respectively. Negative: omitting primary antibody. Magnification 200X.*

6. Discussion

Proteinuria is a common clinical sign in many glomerular and tubulo-interstitial diseases, and it is commonly considered as a risk factor in both cardiovascular and renal disorders. Albumin endocytosis by proximal tubule is a well-known phenomenon, involving the macromolecular complex composed by megalin, cubilin, amnionless (AMN), Dab2 and ClC-5.

Various studies reported albumin uptake in podocytes (Eyre et al., 2007; Koop et al., 2008; Kinugasa et al., 2011; Pawluczyk et al., 2014; Dobrinsky et al., 2014) but nowadays nobody has clarified the mechanism underlying this process, although the presence of megalin, cubilin and ClC-5 was already demonstrated in human podocytes (Prabakaran et al., 2011; Prabakaran et al., 2012; Ceol et al., 2012).

Aims of this study were to explore the presence of the tubular endocytic machinery components listed above also in human podocytes *in vitro*, and to evaluate whether albumin modulates this system. Moreover, we wanted to verify and quantify the expression of ClC-5, megalin and cubilin in both glomerular and tubular compartments in renal biopsies of patients presenting Lupus nephritis and observing the presence of a relationship with clinical data.

Time lapse experiments allowed us to confirm the ability of human podocytes to internalize albumin also at very low doses (10 μg/ml). Several Authors have already demonstrated protein uptake in cultured podocytes but using hundred times higher doses of albumin (range 1.5 - 3 mg/ml) in human or murine cells respectively (Dobrinsky et al., 2015; Eyre et al., 2007). Furthermore, we observed a low percentage (23 ± 9%) of positive cells probably due to the physiological dose of albumin used for these experiments. On the other hand, for the first time we reported the percentage of cells performing albumin uptake. Our results sustain the hypothesis that these cells are committed to protein uptake. Observing podocytes at higher doses of FITC-BSA may lead to an increase in positivity, but this was not possible due to time lapse limitations.

Endocytosis is a well-known process that can occur *via* two different ways: the first is the non-specific binding of the molecule to cell membrane and its subsequent internalization, the second is the receptor-mediated endocytosis which is characterized by a typical saturation kinetic (Hastings et al., 2004). In proximal tubular cells, where the protein uptake machinery is typically expressed, the endocytosis process needs about 30 minutes to take place (Coffey et al, 2015), but in low dose time-lapse experiments we confirmed that human podocytes needed at least 2 hours to display positive vesicles. Moreover, evidences are emerging that podocytes are able to endocytose proteins such as albumin using kinetics consistent with a receptor-mediated process (Pawluczyk et al., 2014; Dobrinsky et al., 2014). In spite of previously reported data which were obtained in murine or urine podocyte-like cells and using higher concentrations of albumin, our results demonstrated that also

at very low doses the endocytosis process is a receptor-mediated mechanism in human podocytes. Moreover, quantifying FITC-BSA endocytosis in human podocytes we highlighted that only a small amount of albumin was internalized, probably due to the physiological range of doses used.

Proximal tubular cells possess a macromolecular complex composed by megalin, cubilin, Dab2, AMN and ClC-5 which is mainly involved in protein uptake from the ultrafiltrate. Moreover, it was previously reported the presence of megalin and cubilin in human podocytes *in vitro* and *in vivo*, and of ClC-5 *in vivo* (Prabakaran et al., 2011; Prabakaran et al., 2012; Ceol et al., 2012). These data support a possible involvement of the tubular protein uptake system also in podocytes. Our results extended the actual knowledge on the presence of the components of the macromolecular system demonstrating not only the expression of megalin and cubilin, but also of ClC-5, AMN and Dab2 in human podocytes *in vitro*. Furthermore, for the first time we disclosed the presence of a co-localization between FITC-BSA and both megalin and cubilin, indicating a possible role for these receptors in albumin internalization by human podocytes *in vitro*.

There is controversy as to whether prolonged exposure to albumin is deleterious to podocytes. Several studies reported an increase in cell death after albumin exposure of murine podocytes but in different experimental designs (i.e. 96 hours' stimulation or doses of albumin higher than 30 mg/ml) (Yoshida et al., 2008; Chen et al., 2011; He et al., 2011). Recently, it was reported that the exposure of human urine derived podocyte-like cells (HUPEC) increased cell death (Okamura et al., 2013). Our experiments did not highlight an increase in cell death, probably because human podocytes taken up less albumin (about a half) than HUPEC.

Our group previously demonstrated the overexpression of ClC-5 in glomeruli of proteinuric patients both at mRNA and protein level suggesting a link between this channel and proteinuria (Ceol et al., 2012). Present data indicate that, in human podocytes, protein overload mainly acts on the expression of the CUBAM complex rather than megalin, with a possible subsequent involvement of the ClC-5 channel in vesicle acidification. To our knowledge, no data are present regarding the expression profile of this system at glomerular level in protein overload *milieu*. Liu et al. reported that, in PTECs, albumin overload induces a slightly increase in megalin and cubilin protein expression at 24 hours, with a marked decrease at 48 hours. The decline was associated with the presence of albumin-induced apoptosis (Liu et al., 2015). In human podocytes, we did not observe a decline in *CUBN* mRNA, probably due to the absence of an increase in cell death and to the low quantity of albumin internalized.

An inhibition of albumin internalization after immunological blocking of cubilin was present, indicating that the uptake mechanism was connected to cubilin even though only a partial inhibition occurred. This can be due to several reasons. It was already

reported the presence of a co-localization between FITC-BSA vesicles and the neonatal Fc receptor (FcRn) in HUPEC (Dobrinskikh et al., 2014) suggesting a possible collaboration between cubilin and FcRn in human podocytes. It is also possible that our antibody may not lead to a complete blocking, thus resulting in a partial inhibition. Similar results were obtained by Nagai et al. that reported the presence of the internalization of Megalin after tagging it with a non-perturbing antibody in canine tubular cells (Nagai et al., 2003). Further studies will be necessary to analyze whether cubilin is the main actor in albumin endocytosis performed by podocytes, in particular using cubilin silenced cells.

Our group previously demonstrated an overexpression of ClC-5 in glomeruli of diabetic nephropathy and membranous glomerulonephritis patients (Ceol et al., 2012). Since in our previous study no correlation between proteinuria and ClC-5 protein expression was observed, we want to investigate the involvement of the protein uptake machinery in several glomerulopathies characterized by different level of proteinuria. In the present study, we focused our attention on patients presenting Lupus nephritis.

In kidney biopsies of SLE patients, we confirmed the presence of megalin, cubilin and ClC-5 also in the glomerular compartment, supporting our observations in human podocytes *in vitro*, and pointing out the importance of the glomerular compartment in albumin handling. Analyzing controls and SLE patients, we did not observe a significant difference in protein levels in both compartments, probably due to the low number of control biopsies and to the high individual variations of both groups. The positive correlation between glomerular and tubular expression of ClC-5, megalin and cubilin, highlighted a relationship between the two compartments independently from proteinuria levels. Moreover, for the first time we demonstrated a strong correlation between megalin and cubilin at glomerular level indicating a cooperation among these two receptors not only in tubular cells.

The unexpected absence of a correlation between ClC-5 and the two receptors in both compartments rise up some questions that can awake further investigations. First, are there any pathways involving ClC-5 independently from megalin and cubilin at renal level? Second, does the pharmacological therapy (ACEi/ARB and/or immunosuppressive drugs) modulate the expression of these molecules? Third, since SLE is an immunological disease with an inflammation pattern on both glomerular and tubular compartments, can this inflammatory damage directly affect the crosstalk between ClC-5, megalin and cubilin?

Interestingly, in SLE patients presenting nephrotic syndrome, we disclose a positive correlation between glomerular ClC-5 and megalin with proteinuria suggesting that this system can respond to protein overload also at glomerular level. These results are in agreement with *in vitro* experiments where we observed an increase in ClC-5,

but we observed a difference between *in vivo* and *in vitro* data on megalin in response to proteinuric *milieu*. We must consider that when we evaluate protein expression in the glomerular compartment, we quantify all the signal present, not only the one produced by podocytes. In fact, in some kidney biopsies we highlighted the presence of ClC-5, megalin and cubilin also in parietal epithelial cells (PECs).

It would be interestingly to increase the number of control biopsies in order to evaluate whether the links found in SLE patients are also present in normal kidney. Moreover, the analysis of other glomerulonephritis such as MCD or Membranous Nephropathy can allow us to distinguish the effects on this system due to the pure glomerular proteinuria rather than the immunological injury.

Since ACEi/ARB and immunosuppressive therapies are known to influence proteinuria levels (Xu et al., 2015; Chan, 2015), we considered the possible influence of these drugs on the expression of ClC-5, megalin and cubilin. We found no significant differences in the expression pattern of these proteins between treated or not treated groups with ACEi/ARB or between treated or not treated with immunosuppressive therapy, while a positive trend was observed at glomerular level evaluating patients without both drugs. It would be interesting to increase the number of these patients in order to better evaluate the effects of the pharmacological treatment on the expression of these molecules.

Podocyte expression of megalin, cubilin and ClC-5 *in vivo* has already been reported (Prabakaran et al., 2011; Prabakaran et al., 2012; Ceol et al., 2012). Here we disclosed for the first time the presence of megalin and cubilin in parietal cells of SLE biopsies. PECs subpopulation are characterizable using specific markers: mature PECs can be identified by the expression of annexin A3 (ANXA3) (Kuppe et al., 2015) and activated PECs can be recognized by the *de novo* expression of CD44 (Smeets et al., 2014). Finally, cells on the Bowman's capsule expressing CD24 are termed adult parietal epithelial multipotent progenitors (APEMP) (Lasagni & Romagnani, 2013).

We confirmed that in SLE kidney biopsies, cells expressing double positivity for megalin and cubilin were mature PECs ($ANXA3^{+}$) presenting a slight positivity also for CD24. This is a new intriguing intermediate phenotype between mature and progenitor cells that present high expression level of both receptors. Since albumin is not the only ligand for both megalin and cubilin (Table 1), it is appropriate to hypothesize a role for these receptors in the uptake of other molecules useful for these hypertrophic cells. Since we found no correlation between the presence of this phenotype and proteinuria, we wonder whether this notable expression can be due to the immunological feature of the disease. Considering that both receptors present immunoglobulin light chains binding sites, this overexpression can be seen as an attempt by PECs to remove antibody excess from the ultrafiltrate. Further studies will be necessary to confirm these hypotheses.

7. Conclusions

In conclusion

- For the first time we demonstrated that human podocytes are committed to perform endocytosis *via* a receptor-mediated mechanism also at very low doses of albumin, supporting their natural ability in doing this function.

- Moreover, we highlighted the presence of the components of the typical tubular protein uptake system such as megalin, cubilin, and for the first time of ClC-5, AMN and Dab-2, also in human podocytes *in vitro*. In addition, protein overload upregulates *CUBN*, *AMN* and *CLCN5* in these cells.

- Functional studies regarding the role of cubilin in albumin uptake underlined its participation in this mechanism even if this is not the only pathway involved. Further studies will be necessary to analyze which may be the partner(s) of the CUBAM complex in this mechanism.

- For the first time we demonstrated the presence of ClC-5, megalin and cubilin in glomeruli of patients with SLE and MCD in addition to controls supporting *in vitro* data.

- In SLE biopsies we highlighted a strong correlation between the two renal compartments in the expression of the protein uptake system, supporting the idea of a partnership between tubular and glomerular cells in albumin uptake *via* the same mechanism of internalization.

- Preliminary data on patients without ACEi/ARB and immunosuppressive drugs lead us to suppose that the pharmacological therapy could effect this system, in particular at glomerular level.

- The differences observed from *in vivo* and *in vitro* data, especially on megalin expression, suggest the involvement of other glomerular cell types in addition to podocytes. In fact, we observed megalin and cubilin expression in PECs of SLE patients.

- The expression of megalin and cubilin in PECs is a real interesting but complex data, since characterization experiments identified a subpopulation with an intermediate phenotype between mature and progenitor cells. Further studies will be performed to better characterize the role of these double-positive cells and the correlation between clinical data and/or disease progression.

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