Glomerular clusterin is associated with PKC-α/β regulation and good outcome of membranous glomerulonephritis in humans

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Mechanisms for human membranous glomerulonepritis (MGN) remain elusive. Most up-to-date concepts still rely on the rat model of Passive Heymann Nephritis that derives from an autoimmune response to glomerular megalin, with complement activation and membrane attack complex assembly. Clusterin has been reported as a megalin ligand in immunodeposits, although its role has not been clarified. We studied renal biopsies of 60 MGN patients by immunohistochemistry utilizing antibodies against clusterin, C5b-9, and phosphorylated-protien kinase C (PKC) isoforms (pPKC). *In vitro* experiments were performed to investigate the role of clusterin during podocyte damage by MGN serum and define clusterin binding to human podocytes, where megalin is known to be absent. Clusterin, C5b-9, and pPKC-α/β showed highly variable glomerular staining, where high clusterin profiles were inversely correlated to C5b-9 and PKC-α/β expression ($P = 0.029$), and co-localized with the low-density lipoprotein receptor (LDL-R). Glomerular clusterin emerged as the single factor influencing proteinuria at multivariate analysis and was associated with a reduction of proteinuria after a follow-up of 1.5 years (−88.1%, $P = 0.027$). Incubation of podocytes with MGN sera determined strong upregulation of pPKC-α/β that was reverted by pre-incubation with clusterin, serum de-complementation, or protein-A treatment. Preliminary *in vitro* experiments showed podocyte binding of biotinilated clusterin, co-localization with LDL-R and specific binding inhibition with anti-LDL-R antibodies and with specific ligands. These data suggest a central role for glomerular clusterin in MGN as a modulator of inflammation that potentially influences the clinical outcome. Binding of clusterin to the LDL-R might offer an interpretative key for the pathogenesis of MGN in humans.

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Membranous nephropathy (MGN) is the major cause of nephrotic syndrome in adults. It requires long-lasting combined therapies with steroids and immune-depressors, and presents variable degrees of progression to end-stage renal failure. In spite of its clinical impact and a lot of research efforts, mechanisms for MGN in humans remain elusive. The bulk of advances on MGN pathogenesis derives from studies on a rat model of Heymann nephritis,[1] which results from the deposition of immunoglobulins (IgGs) in glomeruli following an active immunization with sheep antibodies against rat tubular brush border, Fx1A.[2,3] Components of Heymann subepithelial immunodeposits have been characterized as IgGs, complement fractions (with the formation of the membrane attack complex MAC C5b-9), and clusterin, while the lipoprotein receptor megalin has been identified as their target.[3,4] Therefore, the immune reaction against megalin plays a pivotal role in Heymann nephritis, where complement activation is a crucial determinant of renal injury.

A peculiar role in this context should be brought about clusterin, that is a specific ligand for megalin in several rat

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organs and tissues including glomeruli. Clusterin is a 80 kDa heterodimer of two 40 kDa chains, whose proposed functions include lipid transport, complement defence,[5] and membrane protection.[6] Saunders *et al.*[7] demonstrated that depletion of clusterin enhances immune glomerular injury in perfused kidneys during the autologous phase of passive Heymann nephritis, supporting a protective role of clusterin as host limitation to complement injury. Although the mechanisms of this protective effect have not been elucidated, it has been observed that deficiency of clusterin in mice, due to targeted gene knockout, causes severe renal damage with glomerular immune-deposits.[8]

Clusterin was first isolated from glomerular immune deposits in human MGN in 1988,[9] and then confirmed after more than a decade.[10] Most of the pathogenic issues reported for Heymann nephritis remain, however, undefined in human MGN since the antigen of the autoimmune cascade, megalin, is expressed only by rat glomeruli and not in humans.[11,12] Other antigens for MGN should exist in humans and their characterization is a basic step to define pathogenic elements of human MGN. Results deriving from elegant works by Ronco and co-workers[13,14] on antineutral endo-peptidase represent a good start on this road. The definition of binding sites for clusterin in glomeruli of patients with MGN[10] and the effects on cell signalling pathways could furnish a new key for interpreting the pathogenic cascade of MGN in humans. Based on the available evidence on possible binding of clusterin to other low-density lipoprotein receptors (LDL-Rs) [15] and on complement-induced protien kinase C (PKC) phosphorylation in experimental membranous nephropathy, we started an immune-histochemical analysis of MGN renal biopsies for clusterin, phosphorylated PKC isoforms, and C5b-9. '*In vitro*' studies were conducted to elucidate a possible relationship among clusterin, MGN serum components, and PKC phosphorylation, and to identify a possible binding site for clusterin on human podocytes.

RESULTS

Clusterin is variably expressed in MGN glomeruli

As illustrated in Figure 1, a variable glomerular clusterin expression was observed in MGN biopsies and patients were accordingly subdivided in high (expression level = 2, No. 18), medium (expression level 1, No. 24) and low (expression level 0, No. 18) clusterin profiles. These results were first obtained by utilizing anticlusterin antibodies produced against a peptide of the protein and were confirmed by commercial antibodies.[10] Clusterin was minimally expressed in normal human glomeruli and was almost undetectable in glomeruli of patients with focal segmental glomerulosclerosis (FSGS).[10] Clinical parameters and pathology features at the time of renal biopsy are summarized in Tables 1 and 2. The three clusterin subsets of MGN patients were comparable for age, sex, renal function, lipid profile, proteinuria, histological grading that included MGN class, extension of glomerulosclerosis, and tubulointerstitial damage (Tables 1 and 2). The distribution of therapeutic strategies among groups was comparable as well (Table 1). Finally, serum levels of clusterin were low in almost all patients (mean 205 μg/ml, range 180–310), indicating no correlation of glomerular clusterin deposition with serum levels. A minimum follow-up of at least 18 months was available for 40 patients, again equally distributed among clusterin subsets (2.1, 2.4, and 2.1 years for low, medium, and high clusterin groups respectively). Figure 2 shows the relative changes of proteinuria during follow-up in different subsets of glomerular clusterin expression. In patients with a low clusterin profile, a median increase of 33.6% was observed even if a large variability occurred. In both the other two groups, a clear decrease emerged (−77% for the medium level, −88% for the high level respectively) that was statistically significant ($P = 0.027$, Kruskal–Wallis test). Moreover, multivariate analysis via analysis of covariance two model (Table 3a) confirmed the effect of clusterin on proteinuria. To reduce dispersion, in the latter approach, clusterin was considered present (i.e. medium and high degrees) or absent (low) while, when the first group was split into two levels, only a positive trend was found. Finally, results from the residual regression analysis were consistent both with the univariate analysis and with the analysis of covariance model (Table 3b). A trend to significance was also present for high clusterin profile and low serum cholesterol levels ($P = 0.059$, not shown).

Glomerular clusterin is inversely correlated with pPKC-α/β

In parallel with clusterin expression, renal biopsies were investigated for C5b-9 and phosphorylated PKC isoforms

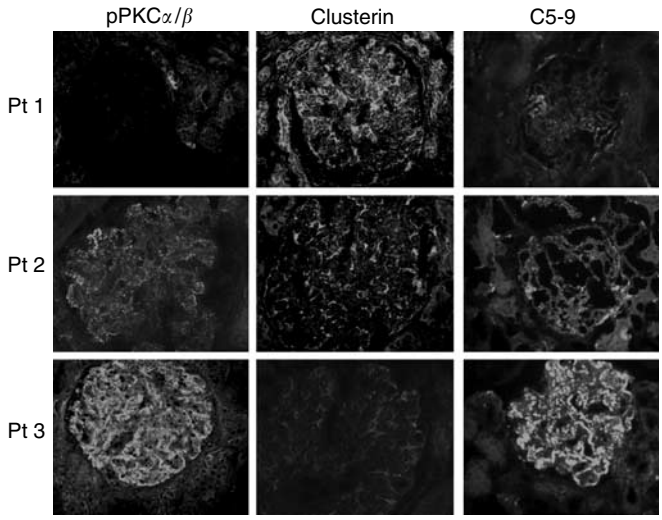

Figure 1 | Clusterin, pPKC-α/β, and C5b9 stainings in renal biopsies of MGN patients. The figure shows the high variability of glomerular clusterin, pPKC-α/β stainings, and C5b9 in renal biopsies from three different MGN patients. Compared to case 1, where a strong clusterin expression is accompanied by the absence of glomerular pPKC-α/β and mild expression of C5b9, case 2 (middle panels) shows moderate staining for all three antibodies. In case 3 (lower panels), a complete negativity of clusterin corresponds to intense pPKC-α/β and C5b9 staining (indirect immunofluorescence, $\times$ 200).

Table 1 | Patients characteristics at the onset by glomerular clusterin grading

Clusterin grade	*N*	Gender		Age (years)	Serum creatinine (mg%)	Serum cholesterol (mg%)	Proteinuria (g/24 h)	Therapy		
		Males *N* (%)	Females *N* (%)	Me (IQR)	Me (IQR)	Me (IQR)	Me (IQR)	A *N* (%)	B *N* (%)	None *N* (%)
Low (grade 0)	18	12 (66.7)	6 (33.3)	59.0 (45.5, 72.5)	1.1 (0.8,1.3)	308.0 (270.0, 338.0)	4.4 (1.5, 7.2)	8 (44.4)	5 (27.8)	5 (27.8)
Medium (grade 1)	24	13 (54.2)	11 (45.8)	63.4 (47.3, 66.7)	0.9 (0.7,1.1)	382.0 (243.5, 412.0)	4.7 (3.5, 6.3)	12 (50.0)	8 (33.3)	4 (16.7)
High (grade 2)	18	13 (72.2)	5 (27.8)	55.4 (47.0, 65.0)	1.0 (0.9,1.1)	302.0 (263.0, 358.0)	5.8 (4.2, 10.0)	7 (38.9)	5 (27.8)	6 (33.3)

N, number of patients; Me, Median value; IQR, Interquartile range.
Data for quantitative variable are given as median and interquartile range due to their angiotensin converting enzyme non-normal distribution. Therapies were grouped in two major subsets, A and B, according to the utilization of steroids or steroids plus immunosuppressors (A) vs angiotensin converting enzyme inhibitors alone (B); few patients received no drugs.

Table 2 | Renal pathology features in patients with MGN subdivided according to glomerular clusterin grading

		Hystology (MGN class)			Glomerulosclerosis (% of glomeruli)			Interstitial fbrosis (grade)			Tubule atrophy (grade)		
	No.	1 *N* (%)	2 *N* (%)	3 *N* (%)	0 *N* (%)	0–20% *N* (%)	>20% *N* (%)	0 *N*(%)	1 *N* (%)	2 *N* (%)	0 *N* (%)	1 *N* (%)	2 *N* (%)
Low (grade 0)	18	6 (33.3)	7 (38.9)	5 (27.8)	15 (83.3)	1 (5.5)	2 (11.1)	10 (55.6)	4 (22.2)	4 (22.2)	10 (55.5)	3 (16.6)	5 (27.7)
Medium (grade 1)	24	5 (20.8)	8 (33.3)	11 (45.8)	19 (79.2)	1 (4.2)	4 (16.6)	12 (50)	11 (45.8)	1 (4.2)	13 (54.2)	11 (45.8)	0
High (grade 2)	18	4 (22.2)	8 (44.4)	6 (33.3)	15 (83.3)	1 (5.5)	2 (11.1)	7 (38.8)	10 (55.5)	1 (5.5)	9 (50)	8 (44.9)	1 (5.5)

MGN, membranous glomerulonephritis.
Glomerulosclerosis was expressed as number of glomeruli with sclerosis (partial or total); interstitial fibrosis and tubule atrophy was given on semi-quantitative basis.

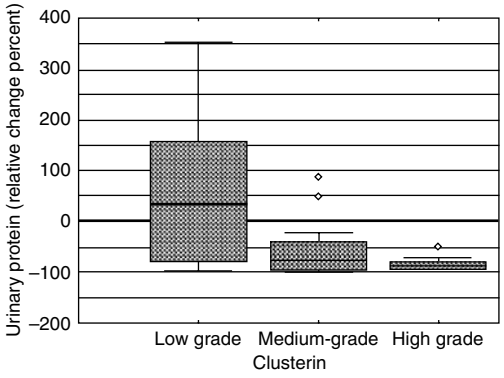

Figure 2 | Correlation between clusterin glomerular profiles and proteinuria during follow-up in MGN patients. Relative percent changes of proteinuria in patients with different subsets of glomerular clusterin expression. The median change of proteinuria was −88 (interquartile range −92.6, −80) in patients with high clusterin profile vs −77 (interquartile range −95, −41) and +33.6 (interquartile range) −78, +157) of the other two groups with intermediate and low clusterin, a difference that was statistically significant at the Kruskal–Wallis test ($P = 0.027$).

(pPKC). As for pPKC, only α and β isoforms were detectable and their expression was variable, from 0-absent to 1-mild-moderate to 2-marked. C5b-9 graded from 0.5-faint to 2-intense.

As represented by Figure 1, pPKC and C5b-9 profiles were inversely correlated to clusterin profiles. The statistical analysis confirmed the inverse correlation of glomerular pPKC-α/β with clusterin in all patients (Sperman's ρ −0.2729 with Prob > $|t| = 0.0319$) (Figure 3), whereas a positive correlation was demonstrated between pPKC-α/β and C5b-9 stainings (Sperman's ρ +0.4527 with Prob > $|t| = 0.0344$).

MGN serum experiments

Based on the above findings, studies were performed to verify PKC phosphorylation in a human podocyte cell line by incubation with sera from MGN patients, and then pPKC was tentatively modulated by clusterin. The following results were obtained by utilizing sera obtained from MGN patients at the time of diagnosis and before the start of any therapy. In total, 10 MGN sera, selected among patients with the highest glomerular expression of pPKC-α/β, were pooled together. Incubation of podocytes with MGN serum produced an intense pPKC-α/β immune-staining (Figure 4j), confirmed by Western blot analysis (Supplementary Information, Figure S1), that was not observed with 10 pooled normal sera (Figure 4d) and five pooled sera from patients with primary FSGS (Figure 4g). Lack of activity of sera from FSGS patients and normal controls seems intriguing since clusterin levels (mean 180 μg/ml, range 165–250) were comparable to those of MGN patients (mean 205 μg/ml, range 180–310).

Instead, preincubation of cells with 200 μg/ml clusterin before the addition of MGN sera completely abolished PKC-α/β phosphorylation (Figure 4m), and the same effect was reached by incubation of cells with de-complemented or Protein-A adsorbed MGN sera.

The results from Protein-A adsorbed sera clearly indicated that soluble factors, most likely antibodies or immune

Table 3 | Effect of clusterin on the variation of proteinuria levels between the onset and the follow-up, adjusted by age, gender, therapeutic approach, and regression to mean

Variables	Regression coefficient	95% CI	*P*-value
(a) ANCOVA model			
Basal level of proteinuria	0.27	−0.14, 0.67	0.187
Presence of clusterin	−0.87	−1.67, −0.08	**0.032**
Therapy			
No treated (reference category)			
A	0.01	−0.91, 0.92	0.990
B	0.40	−0.67, 1.47	0.450
Gender			
Females (reference category)			
Males	0.20	−0.53, 0.93	0.579
Age	0.003	−0.17, 0.23	0.766
(b) Residual model			
Presence of clusterin	−0.74	−1.44, −0.03	**0.042**
Therapy			
No treated (reference category)			
A	0.02	−0.89, 0.93	0.968
B	0.42	−0.64, 1.48	0.424
Gender			
Females (reference category)			
Males	0.27	−0.43, 0.97	0.442
Age	0.004	−0.16, 0.023	0.701

ANOVA, analysis of variance; CI, confidence interval.
Age was inserted as a continuous variable (checking for a linear effect) to avoid the dispersion of values across the cells due to the high number of categorical terms. To take into account possible nonlinear effects, also quadratic and cubic terms were inserted in the model, after centering the original values to avoid co-linearity problems. Nonlinear terms were not included in the final model when not statistically significant.

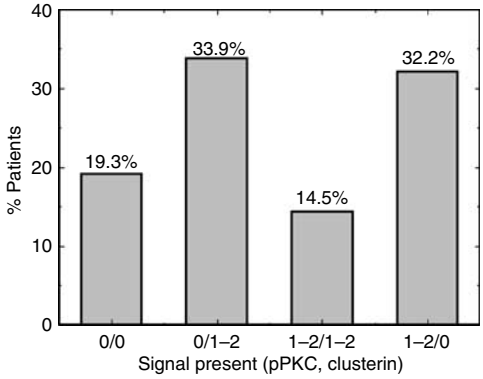

Figure 3 | Relationship between glomerular clusterin and pPKC-α/β in MGN patients. Concordant and discordant stainings for clusterin vs pPKC-α/β in patients with MGN at the onset of the disease. High and medium stainings (semiquantitative value 2 and 1) were compared with absent staining (semiquantitative value 0). The relationship between variables was evaluated by Sperman's coefficient for semiquantitative variables. An inverse correlation was found for concordance between opposite stainings of clusterin and pPKC-α/β (Sperman's ρ −0.2729 with Prob$>|t|=0.0319$). According this approach, discordance (yes/no and no/yes), which defines the inverse correlation between the two variables, identified 66.2% of patients.

complexes, with the participation of complement represent the trigger for *in vitro* pPKC-α/β activation. A most remarkable finding was the prevention of podocyte alterations by clusterin preincubation, which suggests competition of clusterin with some soluble factors removed by Protein-A, and is reached only when clusterin is added alone and in advance.

Clusterin binds to human podocytes and co-localizes with the LDL-R

The presence of clusterin in human MGN glomeruli and especially the block of PKC-α/β phosphorylation in our cell cultures implies clusterin binding and uptake by a podocyte receptor. In the absence of megalin, we hypothesized that another member of the LDL receptor family could act as clusterin receptor in human podocytes. The LDL-R seemed a good candidate because of its presence on human podocytes,[16] and on our human podocyte cell line (Supplementary Information, Figure S2), and because it shares with megalin a similar arrangement of seven complement repeats and one EGF precursor region, an arrangement that seems necessary for the LDL binding to both receptors.[17]

For the experiments, we used biotinilated clusterin (fraction III) purified in our laboratory consisting in a mixture of monomeric and dimeric isoforms of the highest pure grade (Figure 5).

Incubation of biotinilated clusterin with our cells produced a clear podocyte staining (Figure 6a and b) that was not obtained by biotin alone (Figure 6c) and colocalized with the positivity for the LDL-R (Figure 6d–i). When podocytes were cultured in lipoprotein-depleted medium, this treatment produced a doubling of the number of cells stained, without appreciable differences in the quality of labelling in individual cells (Figure 6j).

Inhibition studies were performed by podocyte preincubation with either antibodies against the LDL-R, or competition by receptor associated protein (RAP) (a common ligand of the LDL receptor family members), and both these treatments abolished clusterin positivity (Figure 7a and b). Inhibition controls were obtained by preincubating podocytes with mouse IgG2a antiserum (Figure 7c), or with an antibody directed against β1-integrin, another molecule that is expressed on the podocyte surface and has been implicated in Heymann nephritis, but is not belonging to the LDL receptor family. Both these treatments did not influence the staining of biotinilated clusterin, supporting the feature of specificity of clusterin–LDL-R interaction. These *in vitro* findings seem to be supported by double staining in human glomeruli of MGN patients, where, by sequentially applying antibodies against clusterin and the LDL-R, a yellow merge of the two molecules was clearly evident (Figure 7d–i).

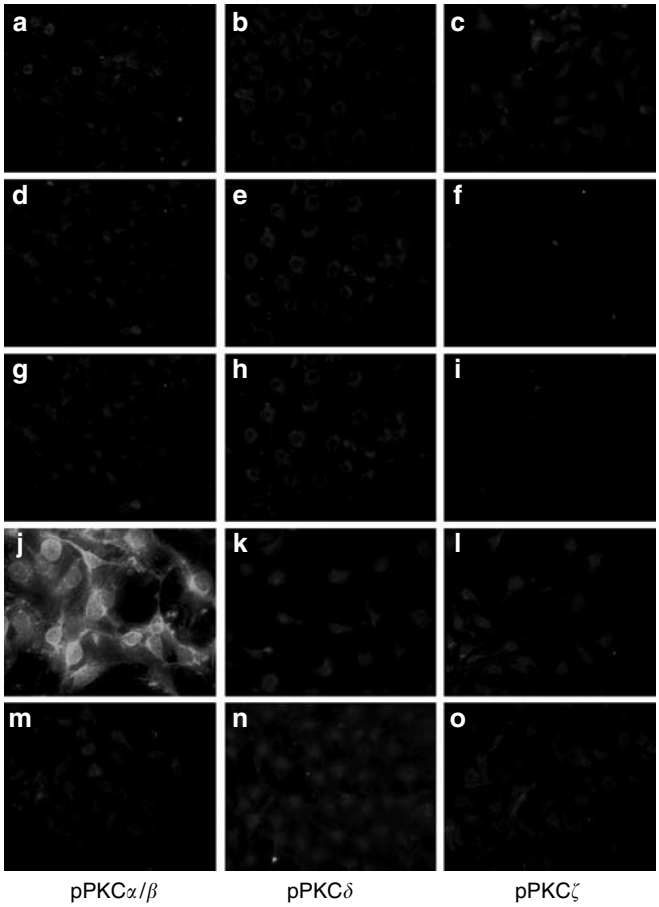

Figure 4 | PKC phosphorylation in human podocytes. The figure shows separate experiments, where glomerular epithelial cells were incubated with (**a–c**) 24 h medium, (**d–f**) 24 h normal serum, (**g–i**) 24 h FSGS serum, (**j–l**) 24 h MGN serum, and (**m–o**) 24 h clusterin, followed by 24 h MGN serum. Cells were then fixed and phosphorylated PKC isoforms α/β (left column), δ (middle column), and ζ (right column), were investigated by immuno-cytochemistry. (**a–i**) Medium, normal serum and FSGS serum did not induce phosphorylation of any PKC isoforms, whereas (**j**) MGN serum produced a diffuse cell staining by the antibody directed against pPKCα/β. (**m**) Preincubation of cells with clusterin prevented the appearance of pPKCα/β. Phosphorylation of δ and ζ PKC isoforms was never detected (indirect immunocytochemistry, $\times$ 250).

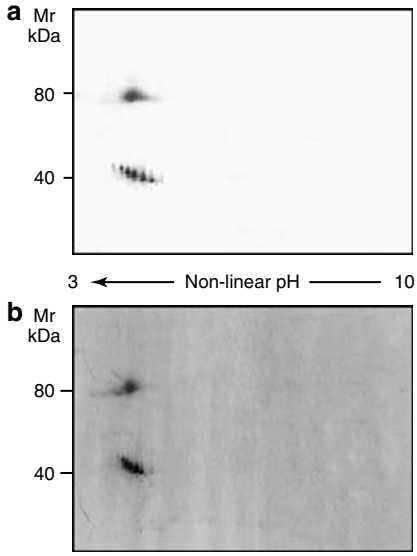

Figure 5 | Characterization of clusterin. Purification of clusterin from normal plasma by a multistep procedure based on precipitation, ultracentrifugation, preparative liquid isoelectric-focusing (Rotofore), and hydroxyapatite chromatography. (**a**) Two-dimensional analysis of fraction III (that was utilized for binding studies) by silver stain (Merril) and (**b**) Western blot with goat-anti human clusterin polyclonal antibodies; horseradish peroxidase conjugated rabbit anti-goat IgG were utilized as second antibodies.

DISCUSSION

In spite of some therapeutic evolution and a bulk of clinical and basic research, the pathogenesis of human MGN remains unknown. Current knowledge on the topics still depends on the description of Heymann nephritis in rats[18,19] that results from formation of immunodeposits in glomeruli following immunization with sheep antibodies against rat tubular brush border megalin. This process requires complement, mostly C5b-9 and also involves the co-precipitation of clusterin in immunodeposits. The characterization of immune-deposits in Heymann nephritis reported for the first time a few decades ago[4] remains a cornerstone for any evolution in our understandings of pathogenesis of MGN in humans, even a key molecule such as megalin is not present in human glomeruli.[3] Clusterin was, instead, demonstrated in glomeruli of a few MGN patients,[9,10] therefore, suggesting co-expression of specific receptor probably sharing some structural motives with megalin. Megalin is a member of the LDR-L family,[20] whose prototype is the LDL-R, that has been recognized on the podocytes surface several years ago[16] and could be bound by clusterin.[15] LDL-R and megalin share some others common ligands such as the so-called RAP, which has been found soon after megalin as a major antigen of passive Heymann nephritis.[21,22] Hiesberger *et al.*[23] demonstrated that several members of the LDL-R family may react with anti-RAP, thus extending the number of possible antigens involved in Heymann nephritis to LDL-R. Our experiments support this possibility, showing specific inhibition of clusterin binding to podocytes by specific LDL-R antibodies. Furthermore, co-localization of clusterin and LDL-R was shown in cultured cells and in biopsy specimens of membranous nephropathy.

Clusterin, also known as apolipoprotein J, is a circulating glycoprotein, which is part of the high-density lipoprotein complex and interacts with a wide range of molecules including itself, lipids, IgGs, and components of the complement membrane attack complex (MAC, C5b-9). Although a great number of efforts to understand its precise role in physiological and pathological situations, the precise effects of clusterin remain not defined even it seems convincing that it acts as a chaperone with protective activities on cells and the results of the present study go in this direction. Clusterin was, in fact, analyzed in 60 MGN kidney tissues that enlarge and precise our previous pilot study,[10] showing that the

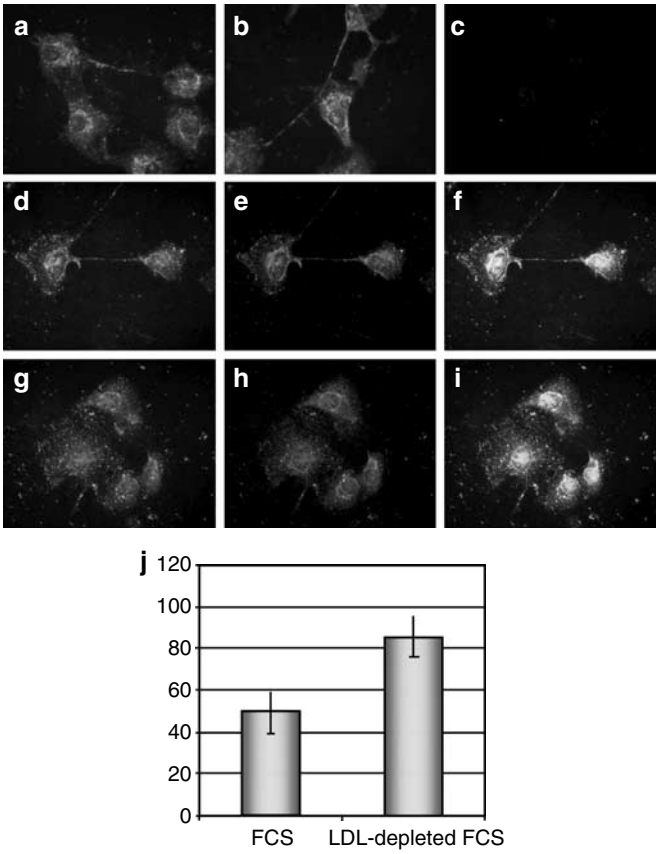

Figure 6 | Clusterin binding experiments. (**a** and **b**) After '*in vivo*' incubation of podocytes with biotinilated clusterin, the molecule is visualized by fluorescent-tagged antibiotin antibodies. (**c**) Biotin alone does not produce any appreciable staining(× 250). (**d, g**) Biotinilated clusterin detection, followed by (**e, h**) anti-LDL-R immunostaining produces an (**f, i**) evident merging of both molecules (× 250, DAPI nuclear counterstaining in blue). (**j**) Compared to standard medium (left bar, FCS), the presence of LDL-depleted medium (right bar, LDL-depleted FCS), determines almost a doubling of the percentage of cells positive for the LDL-R.

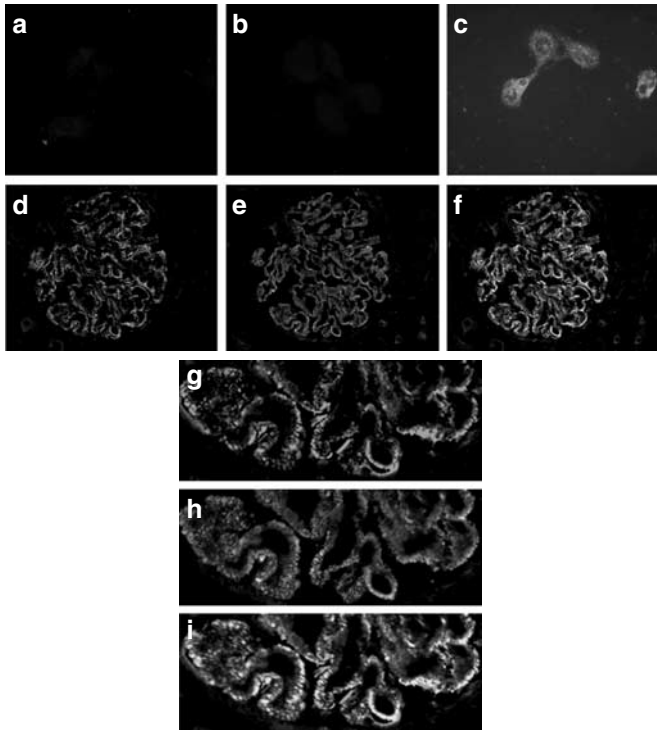

Figure 7 | Binding specificity and co-localization experiments. Preliminary incubation of cells with either (**a**) anti-LDL-R antibody or (**b**) RAP completely prevents clusterin visualization, (**c**) whereas the staining is not abolished when cells are preincubated with nonspecific mouse IgG. (**d**) An MGN case with strong clusterin expression has been stained by anticlusterin antibody, followed by (**e**) anti-LDL-R antibody. (**f**) Colocalization of the molecules is evident in the merging figure (× 200). (**g–i**) At higher magnification, the positivity for (**g, h**) both molecules and their merging along the (**i**) glomerular basement membrane can be observed in detail (× 500, detail).

variable amounts of glomerular clusterin in MGN biopsies are inversely associated with the quantity of proteinuria after a mean follow-up of 1.5 years.

The concept of 'protective clusterin' in immune-mediated diseases is by this time supported by a body of literature evidence. Choi *et al.*[5] demonstrated that clusterin modulates the formation of the complement membrane attack on erythrocytes and inhibits hemolysis.[9,24] As for membranous nephropathy, clusterin null mice develop glomerular changes characterized by diffuse IgGs deposition in mesangial areas and along the glomerular basement membrane.[8] Saunders *et al.*[7] using the complement-dependent isolated perfused rat kidney model of autologous phase passive Heymann nephritis, studied the effect of clusterin depletion on the development of glomerular injury. The authors demonstrated that kidneys perfused with clusterin-depleted serum developed significantly greater proteinuria when compared to control kidneys and showed that C5b-9 was deposited in larger amounts in the clusterin-depleted group.

Here, we show a variation of expression of C5b-9 and pPKC-α/β that inversely correlate to clusterin amounts in glomeruli of MGN patients. It seems therefore that lower levels of clusterin are associated to a more intense activation of the membrane attack complex of complement and a subsequent more intense activation of the PKC pathway. The data induce to hypothesize that lower levels of clusterin are responsible for more severe inflammatory phenomena, and, in agreement with several already published results, suggest the activation of the PKC pathway in podocytes by the membrane attack complex.[25]

In support of this hypothesis, our *in vitro* data show a direct relationship among these phenomena: (a) MGN serum induces a strong expression of p-PKCα/β isoforms. The antibody selected for PKC is specifically directed against a threonine residue (T500) located in the activation loop segment of the molecule that is the phosphorylation site; (b) preliminary addition of clusterin to cells completely prevents pPKC expression, the same occurring if the applied serum is devoid of complement, suggesting that binding of clusterin to the cell surface is able to prevent PKC activation by complement components.

Taken together, the findings above support a key role of clusterin in glomeruli of MGN patients, where the molecule likely acts as a modulator of complement-mediated inflammation and is potentially determinant of the outcome of the disease. We might hypothesize that binding and uptake of clusterin could modify in some way the action of complement and/or immune-complexes, although this possibility remains at present only speculative and has to be supported by further experimental evidence. Given the absence of megalin in human glomeruli, the possible binding of clusterin to the LDL-R offers an alternative mechanism for explaining the action of clusterin in human podocytes.

While we should learn how to regulate clusterin levels in sera, it seems relevant that they inversely correlate with lipid levels,[10] a clear convergence that opens to pharmacologic strategies for human MGN.

MATERIALS AND METHODS

Materials

(see Supplementary materials).

Patients

Eighty-five MGN patients were first enrolled at the following hospitals: Bari, Brescia, Foggia, Genova, Milano, Parma. Among them, 60 cases were selected for the study, based on the following criteria: (a) biopsy-proven diagnosis of idiopathic MGN made between 2002 and 2003. Secondary MGN due to systemic lupus erythematosus. (two cases), hepatitis B virus infection (1 case), and graft-versus-host disease (1 patient) were excluded; (b) availability of adequate (more than 6 glomeruli) frozen kidney samples maintained at $-80C^{\circ}$ for no more than 12 months (c) proteinuria in nephrotic range and (c) absence of any therapeutic approach at time of biopsy. Based on biopsy features, the glomerular stage of MGN was assessed according to Ehrenreich *et al.*[26] Serum was obtained at the time of biopsy and before any therapy was started from 21 patients of this cohort.

After diagnosis, all patients were treated according to different programs: 27 patients received steroids as single drug (1–2 mg/kg) or in combination with immune-suppressors,[27] 18 were treated only by angiotensin converting enzyme inhibitors (Ramipril 5–8 mg/m^2), and 15 had only symptomatic treatments.

Immunofluorescence studies

For immunofluorescence, the unfixed renal tissue was embedded in optimal cutting temperature compound (Tissue-Tek, Bayer, Elkhart, IN, USA), snap-frozen in a mixture of isopentane and dry-ice and stored at -80°C. Subsequently, 5 μm sections were placed on slides and stored at -20°C until immunostained. Cryosections were fixed in cold acetone, rinsed and sequentially incubated with the primary antibody, followed by the proper fluorescent-tagged secondary antibody. For double staining, the procedure was repeated after adequate washing, the material being incubated with the second primary antibody and its specific secondary antibody. After washing, sections were then mounted with an anti-fading mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA). Specificity of labelling was demonstrated by the lack of staining after substituting phosphate-buffered saline and proper control IgGs (rabbit primary antibody isotype control and mouse primary antibody isotype control) for the primary antibody. Sections from the same normal kidney, which showed a mild positivity for clusterin, were included in each set of staining as internal control.

Immunofluorescence results were qualitatively and quantitatively evaluated. Images were acquired by a digital camera (Leica DC 250, Leica Italia, Milan, Italy) connected to a Leitz Diaplan microscope (Leica) and to a Pentium III 500 computer (Maxwel, Rozzano, Italy). Leica IM1000 software was used for image storing and the Leica Q-Fluor software to acquire double staining. Quantitative evaluation was performed by transferring the digitalized images to an electronic image analysis software (ETC3000, Graftek; Villanterio, Pavia, Italy). The system was programmed for glomerular evaluation on selected region of interest (ROI) and an automated macro, composed of a planned threshold procedure, filtering, and Danielsson algorithm, was run on digitalized images. A mean of 7 ± 0.8 glomeruli/biopsy were analyzed. Based on both qualitative and quantitative evaluation of staining, results could be grouped as following: 0 = signal completely absent or very faint; 1 = mild signal involving 80–100% of the glomerular tuft, or moderate staining present in less than 50% of the glomerular tuft; and 2 = intense staining in 80–100% of the glomerular tuft.

Purification of clusterin

Clusterin was purified starting from normal serum high-density lipoprotein according to the technique detailed as Supplementary information. In a first step, serum high-density lipoprotein were isolated by successive precipitation with dextran sulfate and $MGNC_{l2}$ and ultracentrifugation. Clusterin was then purified from the high-density lipoprotein enriched fraction after delipidation and precipitation in tri-*n*-butylphosphate, acetone, methanol[28] by liquid with isoelectric-focusing utilizing the Rotofore^R (Bio-Rad, Hercules, CA, USA) apparatus according to Davidsson *et al.*[29] with some modifications (see Supplementary Materials). Seven chromatography fractions from gel-filtration were labelled from I to VIII and tested for the presence of clusterin by gradient electrophoresis[30] and Western blot with specific antibodies. Fraction III was utilized for experiments with podocyte cell lines *in vitro*. Characterization of fraction III was also carried out with two-dimensional electrophoresis (Figure 5).

Human podocyte cell culture

A line of human podocytes, obtained by infection of primary glomerular epithelial cells with a hybrid Adeno5/SV40 virus as previously described,[31] was used for the experiments. Cells were characterized for the presence of the podocyte proteins nephrin and synaptopodin by immunohistochemistry, Western blot, and RT-PCR (Supplementary Materials, Figure S3). The immortalized cell line was cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose, 10% inactivated fetal calf serum (FCS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C.

MGN serum experiments

To get information on the direct effects of MGN serum on podocytes and the possible effects of clusterin, we designed the following experiments. Ten MGN serum samples, chosen on the basis of glomerular pPKC-α/β expression among those collected before the start of any therapy, were pooled together. Ten normal human serum and five FSGS serum were pooled as well and used as controls. After pooling, clusterin levels were as following: MGN serum (235 μg/ml), normal serum (348 μg/ml), and FSGS serum (188 μg/ml).

Podocytes were alternatively incubated for 24 h with 10% MGN serum, or 10% normal serum, or 10% FSGS serum, or medium alone. After 24 h, cells were extensively washed and either acetone-fixed or collected for protein extraction with the addition of a protease inhibitor. Phosphorylated PKC isoforms were analyzed by both indirect immunohistochemistry and Western blot analysis.

In further experiments, cells were alternatively preincubated for 24 h with 200 μg/ml clusterin before the addition of MGN serum or were incubated with decomplemented (by heating at 56°C for 30 min) or with Protein A adsorbed MGN serum. Clusterin concentration of 200 μg/ml was chosen because it appeared as the minimal effective dose in a preliminary dose-response testing. Protein-A Sepharose CL-4B was used to remove IgGs and immune-complexes from MGN sera. Briefly, 300 μl of Protein-A Sepharose were incubated with 0.5 ml of pooled sera from five MGN patients in 0.05 phosphate buffer pH 7.6 for 2 h at room temperature with gentle agitation. Supernatant after centrifugation at 600 r.p.m. for 15 min at +2°C was used for the experiments. Material bound to Protein-A was eluted with washings in 0.1 M glycine buffer pH2.5.

All the experiments were performed in triplicate and results were evaluated on 300 cells per coverslip.

Two-dimensional electrophoresis and Western blot

(see Supplementary materials).

Clustering binding and uptake assay

Biotin-labelled clusterin fraction III was used to establish the possible binding of the molecule to cultured podocytes. After dialysis against 50 mM carbonate buffer pH 8.6 and 50 mM NaCl, labelling was achieved by incubating 1 mg/ml of purified clusterin with 50 μg/ml water soluble NHS-LC-LC-biotin at 25°C for 60 min.

Cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose and 10% inactivated FCS for 24 h. Following the methods already described[32,33] with modifications, cells were incubated with 20 μg/ml biotinilated clusterin for 2 h at 37°C. After extensive washing, cells were fixed in cold acetone and fluorescent-tagged antibodies against biotin were applied to visualize the bound molecule. Acetone fixation was selected because in our experimental conditions it seemed to allow the best visualization of both surface-bound and internalized clusterin compared to other fixatives. Biotin alone was used with the same procedure as negative control.

LDL receptor assays

Cells were cultured in Dulbecco's modified Eagle's medium containing 25 mM glucose alternatively supplemented with 10% inactivated FCS or 10% lipoprotein deficient serum from fetal calf (Sigma, St Louis, MO, USA), to upregulate LDL-R expression, as previously described,[16] for 24 h. Part of these cells were then washed and collected for protein extraction, and determination of LDL-R was performed by Western blot analysis.

The remaining cells were incubated with 20 μg/ml biotinilated clusterin for 2 h at 37°C, and acetone fixed, then incubated with the mouse monoclonal anti-LDL-R antibody (1 μg/ml). Visualization of clusterin was obtained as described above, whereas the LDL-R positivity was visualized by the secondary antibody Alexa Fluor 546 goat anti-mouse IgG. 4-6-diamidino-2-phenylindole was added to counterstain nuclei. Leica Q-Fluor software was used to acquire double stainings.

Inhibition studies

Following the methods published by Kounnas *et al.*[34] and Agnello *et al.*[33] with modifications, blocking of the LDL receptor was obtained by incubating cells with various dilutions of anti-LDL-R antibody (10–50 μg/ml) for 30 min at 37°C. Another approach was tried by incubating the cells with various dilutions of RAP (Calbiochem, San Diego, CA, USA) (10–50 μg/ml), a common ligand of the LDL-R family. After preliminary experiments, the concentration of 30 μg/ml was chosen for both molecules, because it gave in our hands the maximal inhibitory effect. According to the same published procedures, specificity controls were performed by preincubating podocytes with mouse IgG2a antiserum and an antibody against β1-integrin at the same concentrations, times, and temperatures. Then, biotinilated clusterin was added to the cells, without washing, for 60 min at 37°C, and visualized as above described.

Serum clusterin quantitation assay

Serum clusterin levels were determined with dot blot utilizing commercial anticlusterin antibodies following a procedure developed in our laboratory and already described elsewhere.[10]

Statistics

Descriptive statistics were reported in terms of absolute frequencies and percentages for qualitative data. Medians and the interquartile range were computed as measure of the variability of quantitative variables, due to the non-normal distribution of most of them.

Difference between median values of continuous variables was assessed by the Kruskal–Wallis test, whereas the association between p-PKC, clusterin, and C5b-9 was evaluated by the Spearman's correlation coefficient.

Change in proteinuria concentration between the onset and the follow-up by clusterin degree was evaluated computing the corresponding relative change percent. Moreover, according to Twisk and Proper,[35] both analysis of covariance and residual regression model were applied to adjust for the possible confounding effect of age, gender, type of treatment, and regression to the mean. For this purpose, values of proteinuria concentrations at the onset and at the follow-up were log-transformed and standardized to fulfill the normality distribution assumption of both models. All tests were two-tailed and the conventional P-value of 0.05 was considered the threshold for statistical significance.

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SUPPLEMANTARY MATERIALS

Figure S1.
Figure S2.
Figure S3.

REFERENCES

1. Heyman n W, Lund HZ, Hackel DB. The nephrotic syndrome in rats; with special reference to the progression of the glomerular lesion and to the

use of nephrotoxic sera obtained from ducks. *J Lab Clin Med* 1952; **39**: 218–224.
2. Salant DJ, Darby C, Couser WG. Experimental membranous glomerulonephritis in rats. Quantitative studies of glomerular immune deposit formation in isolated glomeruli and whole animals. *J Clin Invest* 1980; **66**: 71–81.
3. Kerjaschki D, Horvat R, Binder S *et al.* Identification of a 400-kd protein in the brush borders of human kidney tubules that is similar to gp330, the nephritogenic antigen of rat Heymann nephritis. *Am J Pathol* 1987; **129**: 183–191.
4. Kerjaschki D, Ullrich R, Diem K *et al.* Identification of a pathogenic epitope involved in initiation of Heymann nephritis. *Proc Natl Acad Sci USA* 1992; **89**: 11179–11183.
5. Choi NH, Mazda T, Tomita M. A serum protein SP40,40 modulates the formation of membrane attack complex of complement on erythrocytes. *Mol Immunol* 1989; **26**: 835–840.
6. Silkensen JR, Schwochau GB, Rosenberg ME. The role of clusterin in tissue injury. *Biochem Cell Biol* 1994; **72**: 483–488.
7. Saunders JR, Aminian A, McRae JL *et al.* Clusterin depletion enhances immune glomerular injury in the isolated perfused kidney. *Kidney Int* 1994; **45**: 817–827.
8. Rosenberg ME, Girton R, Finkel D *et al.* Apolipoprotein J/clusterin prevents a progressive glomerulopathy of aging. *Mol Cell Biol* 2002; **22**: 1893–1902.
9. Murphy BF, Kirszbaum L, Walker ID *et al.* SP-40,40, a newly identified normal human serum protein found in the SC5b-9 complex of complement and in the immune deposits in glomerulonephritis. *J Clin Invest* 1988; **81**: 1858–1864.
10. Ghiggeri GM, Bruschi M, Candiano G *et al.* Depletion of clusterin in renal diseases causing nephrotic syndrome. *Kidney Int* 2002; **62**: 2184–2194.
11. Kerjaschki D, Farquhar MG. Immunocytochemical localization of the Heymann nephritis antigen (GP330) in glomerular epithelial cells of normal Lewis rats. *J Exp Med* 1983; **157**: 667–686.
12. Zoja C, Benigni A, Verroust P *et al.* Indomethacin reduces proteinuria in passive Heymann nephritis in rats. *Kidney Int* 1987; **31**: 1335–1343.
13. Debiec H, Nauta J, Coulet F *et al.* Role of truncating mutations in MME gene in fetomaternal alloimmunisation and antenatal glomerulopathies. *Lancet* 2004; **364**: 1252–1259.
14. Debiec H, Guigonis V, Mougenot B *et al.* Antenatal membranous glomerulonephritis due to anti-neutral endopeptidase antibodies. *N Engl J Med* 2002; **346**: 2053–2060.
15. Mahon MG, Lindstedt KA, Hermann M *et al.* Multiple involvement of clusterin in chicken ovarian follicle development. Binding to two oocyte-specific members of the low density lipoprotein receptor gene family. *J Biol Chem* 1999; **274**: 4036–4044.
16. Grone HJ, Walli AK, Grone E *et al.* Receptor mediated uptake of apo B and apo E rich lipoproteins by human glomerular epithelial cells. *Kidney Int* 1990; **37**: 1449–1459.
17. Hussain MM, Strickland DK, Bakillah A. The mammalian low-density lipoprotein receptor family. *Annu Rev Nutr* 1999; **19**: 141–172.
18. Feenstra K, van den Lee R, Greben HA *et al.* Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens. I. The natural history: a histologic and immunohistologic study at the light microscopic and the ultrastructural level. *Lab Invest* 1975; **32**: 235–242.
19. Kerjaschki D, Miettinen A, Farquhar MG. Initial events in the formation of immune deposits in passive Heymann nephritis. gp330-anti-gp330 immune complexes form in epithelial coated pits and rapidly become attached to the glomerular basement membrane. *J Exp Med* 1987; **166**: 109–128.
20. Verroust PJ, Christensen EI. Megalin and cubilin – the story of two multipurpose receptors unfolds. *Nephrol Dial Transplant* 2002; **17**: 1867–1871.
21. Farquhar MG, Kerjaschki D, Lundstrom M *et al.* gp330 and RAP: the Heymann nephritis antigenic complex. *Ann NY Acad Sci* 1994; **737**: 96–113.
22. Farquhar MG, Saito A, Kerjaschki D *et al.* The Heymann nephritis antigenic complex: megalin (gp330) and RAP. *J Am Soc Nephrol* 1995; **6**: 35–47.
23. Hiesberger T, Hodits R, Ullrich R *et al.* Receptor-associated protein and members of the low density lipoprotein receptor family share a common epitope. An extended model for the development of passive Heymann nephritis. *J Biol Chem* 1996; **271**: 28792–28797.
24. Jenne DE, Tschopp J. Molecular structure and functional characterization of a human complement cytolysis inhibitor found in blood and seminal plasma: identity to sulfated glycoprotein 2, a constituent of rat testis fluid. *Proc Natl Acad Sci USA* 1989; **86**: 7123–7127.
25. Cybulsky AV, Takano T, Papillon J *et al.* Complement-induced phospholipase A2 activation in experimental membranous nephropathy. *Kidney Int* 2000; **57**: 1052–1062.
26. Ehrenreich T, Porush JG, Churg J *et al.* Treatment of idiopathic membranous nephropathy. *N Engl J Med* 1976; **295**: 741–746.
27. Ponticelli C, Zucchelli P, Imbasciati E *et al.* Controlled trial of methylprednisolone and chlorambucil in idiopathic membranous nephropathy. *N Engl J Med* 1984; **310**: 946–950.
28. Mastro R, Hall M. Protein delipidation and precipitation by tri-n-butylphosphate, acetone, and methanol treatment for isoelectric focusing and two-dimensional gel electrophoresis. *Anal Biochem* 1999; **273**: 313–315.
29. Davidsson P, Puchades M, Blennow K. Identification of synaptic vesicle, pre- and postsynaptic proteins in human cerebrospinal fluid using liquid-phase isoelectric focusing. *Electrophoresis* 1999; **20**: 431–437.
30. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
31. Conaldi PG, Bottelli A, Baj A *et al.* Human immunodeficiency virus-1 tat induces hyperproliferation and dysregulation of renal glomerular epithelial cells. *Am J Pathol* 2002; **161**: 53–61.
32. Birn H, Zhai X, Holm J *et al.* Megalin binds and mediates cellular internalization of folate binding protein. *FEBS J* 2005; **272**: 4423–4430.
33. Agnello V, Abel G, Elfahal M *et al.* Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proc Natl Acad Sci USA* 1999; **96**: 12766–12771.
34. Kounnas MZ, Moir RD, Rebeck GW *et al.* LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell* 1995; **82**: 331–340.
35. Twisk J, Proper K. Evaluation of the results of a randomized controlled trial: how to define changes between baseline and follow-up. *J Clin Epidemiol* 2004; **57**: 223–228.