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Depletion of clusterin in renal diseases causing nephrotic syndrome

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Background. Clusterin is a lipoprotein that has anti-complement effects in membranous nephropathy (MN). In focal segmental glomerulosclerosis (FSGS), it inhibits permeability plasma factor activity and could influence proteinuria. Moreover, with aging, knockout mice for clusterin develop a progressive glomerulopathy with sclerosis.

Methods. Since little is known about clusterin metabolism in humans, we determined clusterin levels and composition in the sera and urine of 23 patients with MN, 25 with FSGS and 23 with steroid-responsive nephrotic syndrome (NS). Renal localization was evaluated by immunofluorescence and morphometry.

Results. Serum clusterin was markedly reduced in active MN, in FSGS and in children with NS compared to controls; after stable remission of proteinuria, nearly normal levels were restored. Among various biochemical variables, serum clusterin was inversely correlated with hypercholesterolemia. Urinary clusterin, representing a 0.01 fraction of serum, was higher in the urine from normal subjects and FSGS patients in remission with proteinuric MN, FSGS and idiopathic NS; clusterin was inversely correlated with proteinuria. In all cases, urinary and serum clusterin was composed of the same 80 kD isoforms. Finally, a decrease in focal segmental or global clusterin staining was found in FSGS glomeruli, especially in areas of sclerosis. Instead, in MN an overall increment of staining was observed that ranged from mild/focal to very intense/diffuse.

Conclusions. The overall pool of clusterin is reduced in glomerular diseases causing nephrotic syndrome, with hypercholesterolemia appearing as the unifying feature. Depletion of clusterin should negatively affect the clinical outcome in nephrotic patients and efforts should be aimed at normalizing clusterin overall pool.

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Clusterin is a ubiquitous protein that is arousing increasing interest owing to its widespread diffusion and multifunctional role. It is composed of two 40 kD subunits (NA1, NA2) encoded by a single gene, which hold together by disulphide bonds [1]. The mature dimer has a molecular mass of 80 kD and is micro-heterogeneous in electrical charge due to glycosylation. This protein has been differently named as apolipoprotein J (ApoJ), sulfated glycoprotein 2 (SGP-2), glycoprotein III (GP-111), testosterone repressed prostate message (TRPM-2), SP-40, 40 cytolysis inhibitor (CL1) and others [2–4]. It is a component of the high-density lipoprotein (HDL) complex with putative functions in the transport of lipids, apoptosis, protection of cells from stress and, most important, in the regulation of complement activity. It has been postulated that clusterin is involved in a variety of human diseases including Alzheimer disease [5], brain injury following ischemia [6] and myocarditis [7]. There is also strong evidence that clusterin plays a role in renal diseases. In fact, clusterin co-localizes with the C5b-9 complex within immune deposits in human membranous glomerulonephritis (MN) [8] where it is supposed to act as a regulator of the C5b-9 cell injury. According to this hypothesis, depletion of clusterin enhances immune glomerular injury in the isolated perfused kidney [9] and clusterin −/− knockout mice are more prone to injury due to immunocomplexes [10].

In an effort to identify the antigen responsible for membranous lesions, in 1995, Orlando, Kerjaschki and Farquhar characterized megalin as the target of the C5b-9 injury in experimental MN [11]. This advance is a cornerstone in the ongoing explanation of the pathogenesis of MN, since megalin is the cell receptor for clusterin in many tissues [12, 13]. According to this mechanistic idea, the presence of clusterin in glomeruli represents a host limitation to complement injury, as it prevents C5b-9 insertion into cell membrane, where the complement com-

Table 1. Clinical details of 23 patients with biopsy-proven membranous nephropathy (MN)

Patient number	Sex	Age	Age at onset	Associated diseases	Histologic stage	Proteinuria *g/day*	Serum clusterin *μg/mL*	Serum creatinine *mg/dL*	Serum cholesterol *mg/dL*	Serum triglycerides *mg/dL*	Treatment
MN (active)											
1	F	14	10	—	1	1.0	172.00	0.5	200	130	ACE I
2	M	51	46	—	1	1.3	142.12	0.7	281	210	MP + Ch
3	M	63	60	—	2	1.3	168.76	0.9	160	215	MP + Ch
4	F	66	60	—	2	1.7	166.20	0.8	201	400	ACE I, MP + Ch
5	M	46	33	—	2	1.9	183.68	1.0	238	197	MP + Ch
6	M	61	58	—	3	2.2	179.75	2.2	368	284	ACE I, Pred
7	M	73	71	—	2	2.2	159.99	1.2	200	210	ACE I, AT1 Comp
8	F	33	14	SLE	4	2.4	236.75	0.8	ND	ND	Pred, statins
9	F	49	46	—	ND	2.7	192.55	0.5	385	168	MP + Ch, ACE I
10	M	61	46	—	2	2.8	280.72	1.2	ND	ND	ACE I
11	M	56	54	—	2	3.2	98.49	1.0	174	82	MP + Ch
12	F	46	40	—	2	3.6	177.00	0.4	386	102	ACE I
13	F	66	60	DM	ND	4.8	158.11	0.8	201	400	ACE I, MP + Ch
14	M	50	49	—	ND	5.0	182.72	1.7	617	770	MP + Ch, AT1 Comp
15	M	54	53	APL, GvHD	3	5.5	245.84	1.7	ND	ND	CSA
16	M	49	24	—	2	6.5	643.59	1.2	270	160	ACE I
17	F	64	62	—	3	9.0	178.40	2.1	297	200	Pred
18	F	35	34	—	4	10.0	98.87	0.7	ND	ND	MP + Ch, cyclophosphamide
19	F	74	72	—	3	13.0	248.16	1.2	ND	ND	ACE I, ACTH
MN (remission)											
20	M	50	45	—	ND	0.1	258.08	0.9	218	160	ACE I
21	M	22	21	—	2	0.1	267.98	0.9	200	140	MP + Ch
22	F	43	38	—	2	0.1	186.25	1.0	244	71	MP + Ch, ACE I
23	M	56	54	—	3	0.4	213.32	1.2	202	145	ACE I, AT1 Comp

Patients were subdivided in two groups, "active" and "remission," according to the entity of 24 hours proteinuria less then 0.5 g/day and higher then 1 g/day. Levels of creatinine and lipids are referred to the day in which clusterin levels had been determined. Therapies refer to drugs taken as a major approach to the disease. Abbreviations are: SLE, systemic lupus erythematosus; DM, diabetes mellitus; APL, acute promyelocytic leukemia; GvHD, graft vs. host disease; Pred, prednisone; CSA, cyclosporine; ACE I, angiotensin-converting enzyme inhibitor; AT1 Comp, angiotensin II type 1-receptor competitor; MP + Ch, methylprednisolone alternated with chlorambucil [17]; ND, not done.

plex competes with clusterin for the access to the same receptor, that is, megalin.

A more recent advance indicates that clusterin also may have some pathogenetic role in focal segmental glomerulosclerosis (FSGS). In fact, clusterin has been recognized as one of the most active physiological inhibitors [14] of the hitherto uncharacterized circulating plasma factor that is the putative cause of the disease [15, 16], and its deficiency should negatively affect proteinuria in this condition.

In accordance with these latter hypotheses, knockout mice for clusterin develop a progressive glomerulopathy characterized by deposition of immunocomplexes that evolve to glomerulosclerosis [10].

To our knowledge, to date no clinical study has addressed the basic point of clusterin levels, renal handling and expression in MN and in FSGS. We therefore evaluated clusterin levels in serum and urine as well as isoform composition and renal expression in three cohorts of patients with nephrotic syndrome caused by different idiopathic glomerular diseases such as MN, FSGS and NS.

METHODS

Patients

Three cohorts of patients who had been enrolled at three nephrology centers in North Italy were studied: 23 patients with MN, 25 patients with FSGS, and 23 patients with idiopathic NS. Fifty normal controls consisted of age-matched subjects of the Hospital Staff and their children. Tables 1, 2 and 3 show the clinical and histological details for each patient including age at onset of proteinuria, associated diseases, renal function and parameters relative to lipids. Therapies performed within six months from enrolment also are reported.

All MN patients were adults who had received a biopsy-documented diagnosis of MN, based on immunofluorescence, light and electron microscopy (Table 1). The glomerular stage was assessed according to Ehrenreich et al [17]. All but two patients had idiopathic MN; one female (no. 8) had MN secondary to lupus erythematosus and another patient (no. 15) received an allogenic bone marrow transplant because of acute promyelocytic leukemia and developed graft versus host disease with subsequent development of MN. Of the 23 patients with MN, 11 had been treated with a six-month course of methylprednisolone alternated with chlorambucil [18], three with oral prednisone, one with adrenocorticoid hormone (ACTH) and one with cyclosporine. Seven patients were given only symptomatic therapy. The definition of activity or remission was done on the basis of 24-hour proteinuria assuming a cut off of 0.5 g/day.

The cohort of FSGS patients consisted of 25 children

Table 2. Clinical details of 18 patients with FSGS and severe protenuria plus six who were in remission at the time of the study

Patient number	Sex	Age	Age at onset	Proteinuria	Serum clusterin	Serum creatinine	Serum cholesterol	Serum triglycerides	Treatment
		years		*g/day*	*μg/mL*	*mg/dL*			
FSGS (active)									
1	F	6	4	13.0	142.90	0.7	459	540	Pred, CsA, ACE I, statins
2	F	75	75	6.5	105.32	1.3	458	168	Pred, statins
3	M	17	10	16.5	128.81	3.5	583	377	ACE I, statins
4	M	23	8	10.0	221.37	1.0	184	95	Pred, statins
5	M	16	8	1.0	78.23	0.9	137	59	Pred, statins
6	M	16	8	2.4	504.76	0.9	170	77	Pred, statins
7	F	11	4	8.2	160.58	7.0	119	517	ACE I, statins
8	F	51	36	0.6	232.32	6.0	138	150	ACE I, statins
9	M	6	3	14.0	133.79	0.7	325	305	Pred, ACE I, statins
10	F	30	25	1.2	178.95	0.6	235	324	ACE I, AT1 Comp, statins
11	F	7	3	1.4	159.20	0.4	618	383	Pred, ACE I, statins
12	M	43	30	2.9	118.90	1.2	250	180	Pred, CsA, statins
13	M	15	2	3.8	145.20	1.0	236	114	Pred, CsA, statins
14	M	31	26	18.7	204.69	0.6	351	404	ACE I, statins
15	M	66	50	1.2	191.40	1.3	121	120	Pred, ACE I, statins
16	M	17	8	3.8	155.95	1.1	178	116	Pred, CsA, ACE I, statins
17	M	16	7	1.2	160.58	2.6	192	176	ACE I, statins
18	F	47	46	2.9	236.65	0.6	357	145	Pred, statins
19	M	8	4	2.3	185.13	0.5	289	96	CsA, statins
FSGS (remission)									
20	M	18	7	0.4	343.70	0.6	139	87	CsA-previous Pred
21	M	12	5	0.1	498.47	0.6	218	106	CsA-previous Pred
22	M	15	8	0.1	268.21	1.0	193	129	Pred, CsA
23	M	17	8	0.2	258.17	1.0	154	150	Pred, CsA—previous Cycl
24	M	8	3	0.1	119.30	1.0	147	80	Nothing—previous Pred, Cycl
25	M	8	4	0.1	393.60	1.0	118	70	Nothing—previous Pred

Levels of creatinine and lipids refer to the day in which clusterin levels had been determined. Therapies refer to drugs currently in use or that had been the major therapeutic approach. 'Previous' indicates that the treatment had been carried out at least 6 months before this study. Abbrevations are: Pred, prednisone; CsA, cyclosporine; ACE I, angiotensin-converting enzyme inhibitor; Cycl, cyclophosphamide; AT1 Comp, angiotensin II type 1-receptor competitor.

Table 3. Clinical details of 23 children with idiopathic nephropathic syndrome who were proteinuric at the time of the enrollment, but responded to steroids and/or cyclosporine

Patient number	Sex	Age	Proteinuria	Serum clusterin	Serum creatinine	Serum cholesterol	Serum triglycerides	Treatment
		years	*g/L*	*μg/mL*	*mg/dL*			
Idiopathic NS								
1	M	8	15	466.16	0.4	218	119	Pred, statins
2	M	7	2	238.25	0.3	263	98	Pred, CsA, statins
3	M	6	3.9	144.30	0.3	327	330	Pred, statins
4	M	8	2.5	173.06	0.3	114	236	Pred, statins
5	M	10	1	214.79	0.9	170	170	Nothing
6	F	5	6	564.11	0.6	267	155	Pred, statins
7	F	4	30.6	155.29	0.4	114	46	Pred, statins
8	F	3	10	124.57	0.5	492	369	Pred, CsA, statins
9	M	4	0.93	198.27	0.4	449	220	Pred, statins
10	M	9	0.89	360.00	0.4	314	176	Pred, statins
11	M	7	1	138.92	0.5	269	106	Pred, statins
12	M	9	1.7	159.39	0.5	229	162	Pred, statins
13	F	5	13	142.90	0.3	417	299	CSA, statins
14	M	5	3	166.70	0.3	309	60	Pred, statins
15	F	8	10	147.27	0.7	491	46	Pred, statins
16	M	4	1.3	150.27	0.4	273	68	Pred, statins
17	M	5	7.8	181.25	0.4	456	374	CsA, statins
18	M	9	10	140.71	0.7	369	412	Pred, CsA, statins
19	F	5	2	431.02	0.3	180	110	Pred, statins
20	F	7	5	121.52	0.4	425	209	Pred, statins
21	M	6	4	173.35	1.1	142	108	Pred, statins
22	F	14	4	120.24	0.5	466	121	Pred, statins
23	M	10	10	130.24	0.6	337	181	CsA, statins

The onset of proteinuria occurred in all these cases within 6 months from the enrollment. Sera for clusterin and lipid determination were obtained during the active phase of the disease. Abbrevations are: Pred, prednisone; CsA, cyclosporine.

and adults with drug-resistant nephrotic syndrome who had undergone renal biopsy and had received a diagnosis according to the pathological criteria of the presence of at least one area of segmental or global sclerosis in glomeruli with an expansion of extracellular matrix (Table 2). At the time of enrollment 19 patients presented with a full-blown picture of nephrotic syndrome that did not respond to steroids (2 mg/kg for 2 months plus 4 pulses with 10 mg/kg). Nine patients were treated with steroids (2 mg/kg for 60 days) that was associated with cyclophosphamide in two. Nine further patients were treated with cyclosporine (5 mg/kg as the starting dose). Six further children with the pathological diagnosis of FSGS had responded to cyclosporine and four were still being given the drug at the time of this study. Two children had stopped the treatment two years earlier and had normal urinalysis and renal function without using any drug.

The third group consisted of 23 children who presented with nephrotic syndrome at the time of the enrollment but who responded to a cycle of therapy with steroids (2 mg/kg for 2 months) or steroids plus cyclosporine (5 mg/kg initial dose). For this reason, they were classified as having idiopathic NS (Table 3). To comply with standard clinical practice for a good outcome, none in this group was studied with renal histology.

Serum and urine studies

Serum was obtained in the morning, after an overnight fast. Blood was immediately centrifuged at $1500 \times g$ for 15 minutes at room temperature and stored at -80°C for less than one month until assayed for clusterin. The first morning micturition was collected in sterile tubes and immediately centrifuged and stored at -20°C.

Purification of clusterin for standards

Human clusterin was purified from serum by immunoadsorption using the previously mentioned purified polyclonal antibodies coupled to CNBr-activated Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and concentration was determined by absorbance measurement at 280 nm with a 1 cm cell using E 70073 $mol/L^{-1} \cdot cm^{-1}$.

Anti-clusterin antibodies

Affinity purified goat anti-human clusterin antibodies were purchased from Chemicon (Temecula, CA, USA). They were utilized for dot-blot analysis. The specificity of these antibodies was tested by two-dimensional electrophoresis and Western blot analysis (described later in this section) with whole human serum. In reducing conditions, they recognized two single spots corresponding to the reduced subunits (NA1, NA2; data not shown), while in the absence of reducing agents, these antibodies recognized a single isoform with a molecular weight of 80 kD and a pI between 4.6 and 4.9 that corresponded to the mature dimer (Fig. 1).

Further polyclonal antibodies for immunofluorescence, were produced by injecting the following peptides of the α subunit of clusterin in rabbits: (*1*) NH_2-QHPPTEFIR EGDDDR (268-282); (*2*) NH2-RRELDESLQVAERLT RKY (324-341); (*3*) NH2-LTQGEDQYYLRVTT (375-387). Specificity of antibodies was controlled by immunoblot and two-dimensional electrophoresis of whole serum and urine (two-dimensional electrophoresis is described later in this section; Fig. 1).

Preparation of antibody-enzyme conjugate

Two hundred micrograms of the above commercial polyclonal antibodies to clusterin were coupled to horseradish-peroxidase (EC 1829696) according to specific information given by the peroxidase labeling kit (Roche, Mannheim, Germany). The conjugate was mixed with an equal volume of glycerol for storage at -20°C.

Clusterin quantification by dot blot

All samples and the calibrator serum were diluted with Tris buffered saline (TBS) pH 7.4 (1:10 vol/vol). A calibration curve was prepared by dilution of purified clusterin in TBS at the programmed levels of 25, 50, 100, 200, 400 and 800 μg/mL. Sera and urines were diluted in the same buffer to achieve the desired range of levels, within the standard curves.

For clusterin determination we utilized a Bio-Dot apparatus (Bio-Rad, Hercules, CA, USA) following the instruction manual with minor modifications. Accordingly, the nitrocellulose membrane was pre-wetted in TBS and placed on a sheet of Whatman 3 mm filter paper embedded with the same solution. After removal of the air bubbles trapped between the two sheets by gentle pressure, the sample template was placed on the nitrocellulose membrane and a vacuum was applied for a few minutes, to fill up the 96 sample wells with 50 μL using a multi-channel pipette. The vacuum was applied until all the samples were adsorbed. The same operation was repeated five times with 150 μL of buffer each, to wash out the non-adsorbed sample. The nitrocellulose was then gently removed.

Hybridization was preceded by incubation with a blocking solution of 15% polivinyl-pyrrolidone mixture (10,000 and 40,000 molecular wt) in 0.1 mol/L acetic acid for one hour at 37°C, which then was removed by washing in 20% Tween-TBS. Incubation with the HPR–anti-clusterin antibodies complex 0.5 μg/mL in 1% bovine serum albumin (BSA) in TBS was performed for two hours at room temperature.

The membrane was then washed four times, 15 minutes each, with TBT-T prior to developing the immuno-

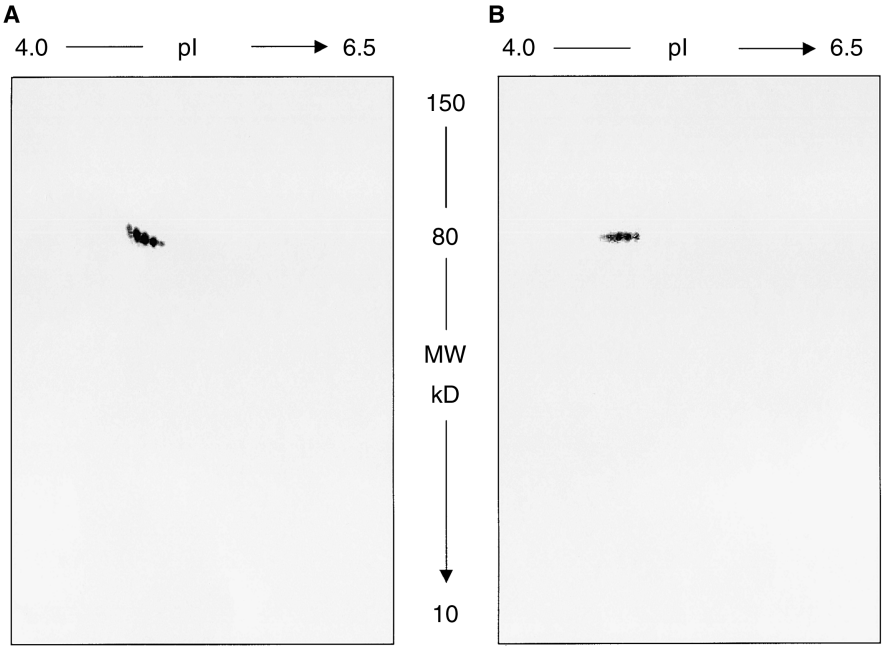

Fig. 1. Two dimensional electrophoresis in a non-reducing condition and immunoblotting with commercial polyclonal antibodies against clusterin of serum (A) and urine (B) from a patient with membranous nephropathy. Abbreviations are: pI, isoelectric points; MW, molecular weight.

blot with the enhanced chemiluminescence reagent ECL-Plus kit (Amersham Pharmacia Biotech). The detection of fluorescent signals was acquired with Optical Scanner Storm 860 with excitation λ 420 nm and emission λ 460 nm.

Validation of the assay was done by determining clusterin levels in a standard and in three normal sera at different dilutions (Fig. 2). Coefficients of variation were calculated by repeating clusterin determination at five different dilutions in ten sera.

Isoform composition of clusterin in serum and urine

Clusterin in serum and urine was characterized by two-dimensional electrophoresis and immunowestern blot. Two-dimensional electrophoresis preparation, rehydration of immobilized polyacrylamide gradients (IPGs), and polyacrylamide electrophoresis (PAGE) have been described in detail elsewhere [19, 20]. Briefly, the IPG strips were rehydrated overnight at 4°C in 9 mol/L urea, 2% wt/vol CHAPS; 0.6% wt/vol carrier ampholytes with a wide range mixture (60% pH 4 to 8, 40% pH 3.5 to 10) and a trace of bromophenol blue. Proteins, 30 μg, were solubilized with a solution containing 9 mol/L urea, 4% wt/vol CHAPS and 40 mmol/L Tris. Isoelectric focusing was performed at 18°C. The pH gradient was created with Immobilines (IPGs; Amersham Pharmacia Biotechnology) pH between 3 and 10 in the first dimension; SDS-PAGE in the second dimension was performed following the original technique described by Bjellqvist et al [20]. The applied voltage for electrophoresis was increased from 300 to 3500 V during the first five hours, followed by 5000 V for a total of 100 kV · h. Before the two dimensional run, IPG strips were equilibrated within the strip tray for 30 minutes with a solution of 0.05 mol/L Tris-HCl buffer pH 6.8, 6 mol/L urea, 30% vol/vol glycerol, 2% wt/vol SDS and a trace of bromophenol blue. The second dimension was performed on 180 × 160 × 1.5 mm slabs of polyacrylamide gradient gels (% T 8-16) using piperazine diacrylamide (PDA) as the cross-linking agent. The gels were run at 45 mA/gel constant current and maintained at a temperature of 12°C.

For Western blotting, proteins were transblotted to Hybond nitrocellulose membranous (Amersham Pharmacia Biotech) with a Novablot semidry system using a continuous buffer system with 38 mmol/L Tris, 39 mmol/L glycine, 0.035% SDS and 20% methanol. The transfer was achieved at 1.55 mA/cm^2 for 3.5 hours.

Immunofluorescence

Clusterin expression in the kidney was studied by indirect immunofluorescence in renal biopsies from 22 patients (10 with idiopathic membranous nephropathy and 12 with idiopathic segmental sclerosis) and, for comparison, in 5 cadaver kidneys that could not be grafted because of vascular abnormalities.

Tissue samples for light microscopy were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Routine staining was performed according to standard techniques. For immunofluorescence, the unfixed renal tissue was embedded in OCT compound (Miles Scientific, Naperville, IL, 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 immunostaining.

Briefly, cryosections were fixed in cold acetone, rinsed,

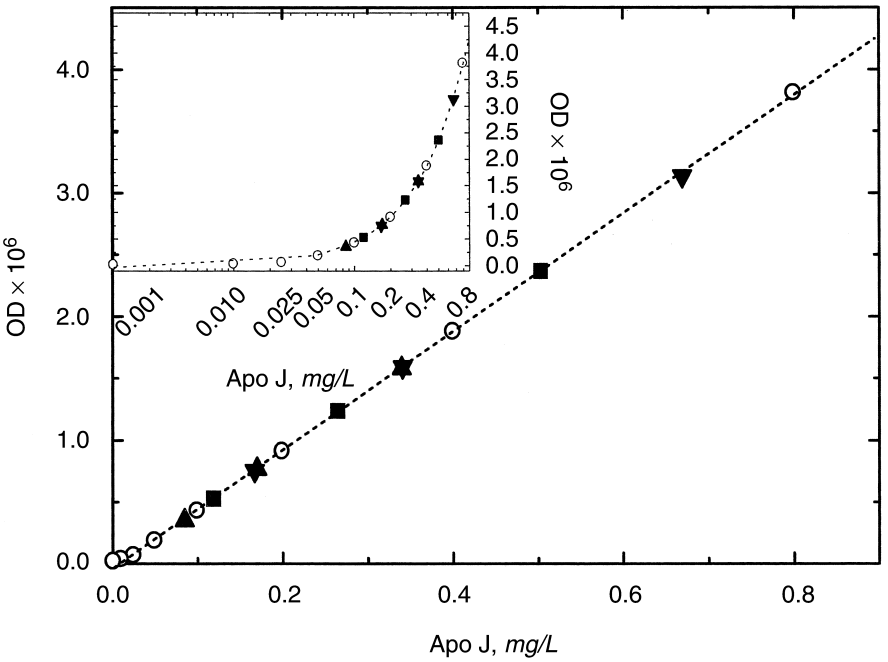

Fig. 2. Calibration curve with a purified standard of clusterin and three sera at different dilutions. The average optical density for the chemiluminescence analysis was plotted against the amount of clusterin loaded. Symbols are: (○) ApoJ St; (▲) serum 1; (▼) serum 2; (■) serum 3. (Inset) A plot that utilizes a logarithmic scale in the abscissa to visualize the curve at low concentrations.

and sequentially incubated with the primary rabbit polyclonal antibody against clusterin, followed by fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit secondary antibody (Histoline; Zymed, Milan, Italy). After washing, sections were then mounted with an anti-fading mounting medium (Vectashield; Vector, DBA Italia SRL, Milan, Italy). Specificity of labeling was demonstrated by the lack of staining after substituting phosphate buffered saline (PBS) and proper control immunoglobulins (Zymed) for the primary antibody.

Immunofluorescence results were evaluated by an electronic image analysis system (ETC3000, Graftek; Villanterio, Pavia, Italy). Images were digitalized using a video camera (DC200; Leica, Milan, Italy) connected to a Leitz Diaplan microscope (Leica) and to a Pentium III 500 computer (Maxwel, Rozzano, Italy) equipped with a frame grabber (Leica). The electronic system was programmed for glomerular evaluation by selecting the ROI (region of interest mean statistic) analysis and an automated macro, composed of a planned threshold procedure, filtering and Danielsson algorithm, was applied to all digitized images. A mean of 8 ± 1.2 (minimum 6 to maximum 19) glomeruli/biopsy were analyzed. Globally sclerotic glomeruli were excluded from the evaluation. Results were expressed as number of positive pixels/glomerular area, automatically exported and elaborated in an electronic file (SPSS 9.0 for Windows; SPSS Inc., Chicago, IL, USA) using the mean and standard deviation. The significance was set at $P < 0.05$ and was analyzed using the χ^2 test.

Statistical methods

The serum concentrations of clusterin in MN, FSGS, idiopathic NS and controls were compared utilizing the one-way analysis of variance (ANOVA). Data are given as mean ± standard deviation.

RESULTS

General features

This study was conducted in two cohorts of patients with histologically-proven MN and FSGS (Tables 1 and 2) and in a further group of children with nephrotic syndrome who responded to classical treatments (Table 3). Overall, most of them were frankly proteinuric while only few presented mild proteinuria or were in remission. In fact, among the MN patients only four had proteinuria less than 0.5 g/day, six were frankly proteinuric (>5 g/day) and the remaining thirteen showed levels between 1 and 5 g/day. Renal function was normal in all but four subjects who presented a creatinine level between 1.7 and 2.2 mg/dL. Also, FSGS patients presented variable proteinuria: six were in stable remission (proteinuria <0.5 g/day), eight were frankly proteinuric (>5 g/day) and eleven presented intermediate levels between 1 and 5 g/day. Four out the six FSGS patients in remission were still receiving drugs at the time of enrollment, while another two had drug-induced remission two years before and during the study period were without any treatment. Serum creatinine levels were normal in 20 out of the 24 patients of the FSGS group; in four patients (1 child, 2 adolescents and 1 adult) creatinine had reached

Table 4. Serum and urinary levels of clusterin in the cohorts of patients with membranous nephropathy and focal glomerulosclerosis subdivided according to levels of proteinuria in active disease (>1 g/day) and remission (<0.5 g/day)

Group	N	Clusterin serum μg/mL	Clusterin urine μg/mL	Creatinine μg/mg
Controls	50	366.60 ± 62.0	7.06 ± 0.42	7.8 ± 0.47
MN (active)	19	220.77 ± 110.9[a]	1.03 ± 0.75[a]	1.47 ± 1.07
MN (remission)	4	236.60 ± 38.7[a]	3.31 ± 5.65	3.68 ± 6.28
FSGS (active)	19	181.44 ± 89.0[a]	1.32 ± 2.34[a]	2.2 ± 3.9
FSGS (remission)	6	312.50 ± 130.5	5.27 ± 6.55	6.59 ± 8.2
Idiopathic NS	23	210.11 ± 122.0[a]	0.72 ± 0.99[a]	1.44 ± 1.98

A group of 23 children with idiopathic nephrotic syndrome who were successively found to respond to steroids or cyclosporine was enrolled to address a specific role of proteinuria in clusterin levels. Urinary clusterin was determined on fresh samples obtained in the morning and results are given as concentration per mL or corrected for urinary creatinine.

[a] $P < 0.001$ vs. controls

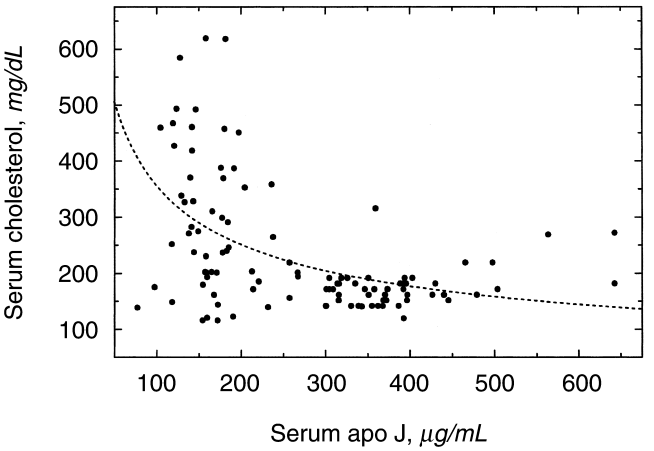

Fig. 3. Inverse correlation between serum clusterin levels and cholesterol in the entire cohort of 72 patients with nephrotic syndrome (MN, FSGS, NS) and 50 controls. The former group was mainly comprised of patients with an acute disease, with a minor number of patients with a remittance of proteinuria. $y = a X^b$, where $a = 3605.07$, $b = -0.5032$; $r = .049$; $P < 0.001$.

levels of end-stage renal failure (Table 2). The lipid profile was variable and in all cases it was strictly dependent on the presence of nephrotic syndrome. Data on children with idiopathic NS are reported in Table 3. Proteinuria and lipids also were variable in these cases with most patients presenting with heavy proteinuria and hypercholesterolemia. All of the children belonging to this group responded to steroids or to an association with cyclosporine (**Methods** section) and after that serum was obtained for determination of clusterin and other parameters.

Serum and urine clusterin

Figure 1 demonstrates the specificity of the antibodies utilized in the dot blot assay. Figure 2 shows the high reproducibility of our new technology based on dot-blot analysis of clusterin; accordingly, the coefficient of variation at any given point of the calibration curve was less than 5%. Based on these results that validated the new technology, we determined clusterin levels in serum and urine of the three groups of patients enrolled in the study: MN, FSGS and idiopathic NS. The results are separately reported in Tables 1, 2 and 3. The means of serum levels for each group, together with urine concentrations, are reported in Table 4. Serum clusterin was significantly lower than in controls (366.6 ± 62 μg %) in all the cohorts of patients presenting with proteinuria higher than 1 g/day The following serum levels were observed: (*a*) active MN, 221.77 ± 110.9 μg/mL; (*b*) active FSGS, 181.44 ± 89 μg/mL; (*c*) idiopathic NS, 210.11 ± 122 μg/mL. In FSGS patients with stable remission, serum clusterin was only mildly lower than in controls (312.5 ± 130.5 μg/mL). Prior determinations during proteinuria in four of these patients demonstrated low levels; however, the small number of patients in this group does not allow any conclusion except that there is a trend towards normalization upon remission.

Seeking possible regulators of the clusterin levels in NS, we tried to define correlations with a few biochemical parameters and found a highly statistical inverse one with the cholesterol levels (Fig. 3).

As shown in Table 4, the concentration of clusterin in urine was lower than in serum, roughly corresponding to a 0.01 fraction with some difference between normal versus proteinuric urine. In fact, urinary clusterin was higher in normal controls (7.06 ± 0.42 μg/mL) than in patients with active MN (1.03 ± 0.75 μg/mL), active FSGS (1.32 ± 2.34 μg/mL) and with idiopathic NS (0.72 ± 0.99 μg/mL). In the small group with non-active FSGS the urinary levels were variable, but the trend was to reach the upper values observed in the control population (5.27 ± 6.55 μg/mL). Overall, urine clusterin levels were inversely correlated ($P < 0.001$) with proteinuria (not shown). Finally, the results of immunowestern blot of serum and urinary clusterin presented in Figure 1 excluded a selective renal excretion, since the isoform with 80 kD, that is, the mature dimer, is present in both fluids.

Immunofluorescence

Tissue studies were performed in order to define whether renal deposits occur during the pathological process leading to MN and FSGS. While data on clusterin renal deposition are already available for MN [8], to date no study has addressed the problem in FSGS. Our results are given in Figures 4, 5 and 6. Immunofluorescence with peptide-produced antibodies showed diffuse glomerular deposits confined to podocytes in normal kidneys (Fig. 4A). In the interstitium (Fig. 4B), some tubuli also showed positive staining. An overall statistically significant ($P < 0.05$) increment of staining was observed in MN glomeruli (Fig. 5); the staining was variable in intensity and

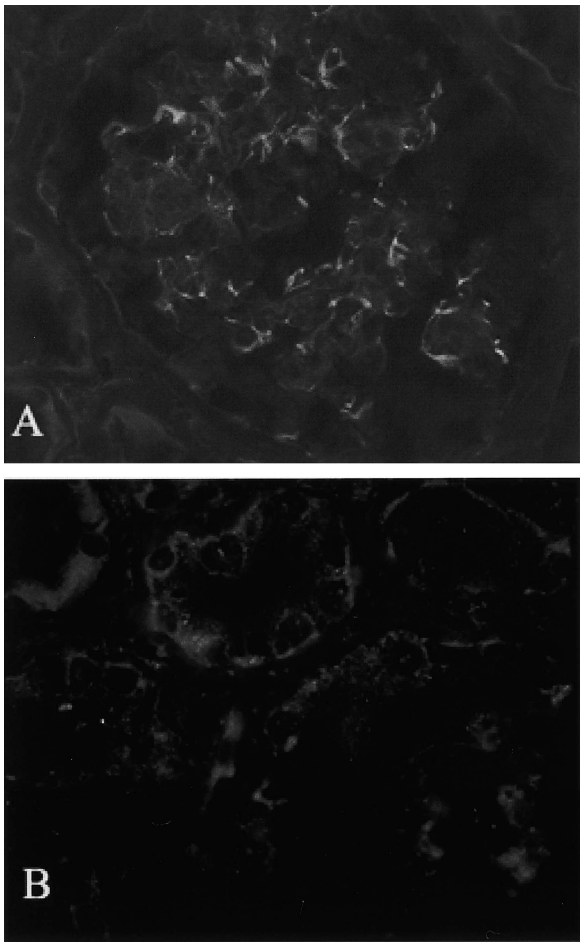

Fig. 4. Normal kidney showing clusterin staining present on glomerular podocytes (*A*) and on some tubules in the interstitium (*B*) (IF, ×400). Immunofluorescence (IF) was done with antibodies raised against clusterin peptides.

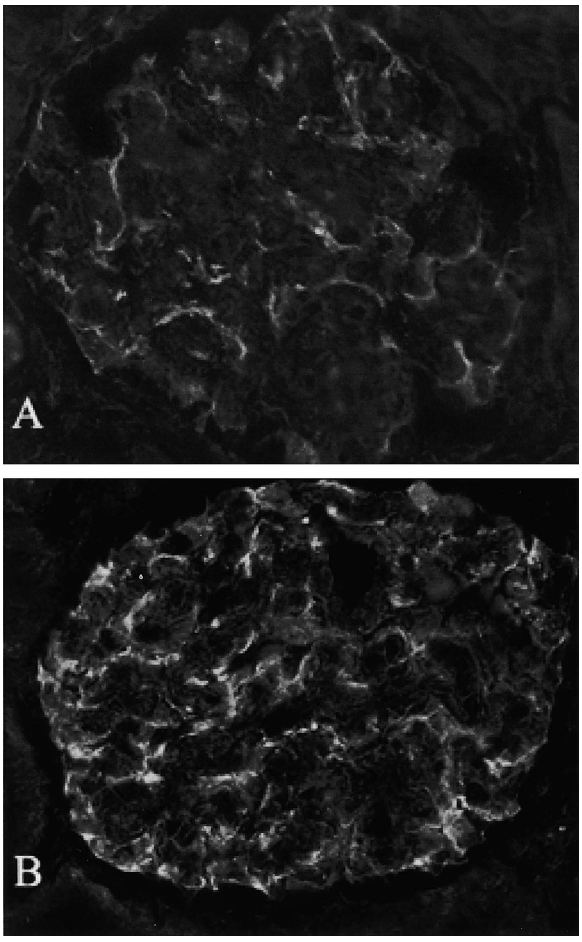

Fig. 5. Membranous nephropathy showing that the increment in clusterin staining is variable in different cases from moderate (*A*) to very intense (*B*) (IF, ×400). Immunofluorescence was done with antibodies raised against clusterin peptides.

diffusion from mild, focal and segmental (Fig. 5A) to very intense, global and diffuse (Fig. 5B).

A focal, but not statistically significant decrease of clusterin staining was detected in FSGS (Fig. 6). In addition to the glomeruli maintaining a staining similar to normal kidney (Fig. 6A), a global (Fig. 6B) or segmental (Fig. 6C) staining absence was found in 30% of glomeruli. When segmental, it was mainly but not exclusively detected in sclerotic areas.

No differences from normal kidney were observed in terms of tubular staining in both MN and FSGS patients.

The results of the quantitation of clusterin deposits by morphometric analysis are given in Figure 7. MN kidneys present a statistically significant mean increase.

DISCUSSION

Clusterin is thought to play an important protective function in specific renal diseases such as MN and FSGS, where it should act at different levels with each type of renal disease. In MN, clusterin competes with C5b-9 for the same receptor, that is, megalin in podocytes [11–13], and limits to some extent the accessibility and the proteolyses activity of the complement at this level. It is not surprising that serum depletion of clusterin represents a negative event in experimental models of MN [9] and in animals lacking the protein due to genetic manipulation [7]. In fact, after depletion of clusterin, glomerular deposits of complement and immunoglobulins are wider and proteinuria is significantly higher than in non-depleted animals. Moreover, clusterin-deficient mice develop a progressive glomerulopathy with age, characterized by immunocomplex localization in mesangium and glomerulosclerosis that mimics, in some way, FSGS in humans [10]. The possibility that clusterin plays a protective role in FSGS also is supported by in vitro studies showing an increased permeability activity in serum of patients with FSGS when the serum is depleted of clusterin [14]. Since permeability activity in FSGS is linked

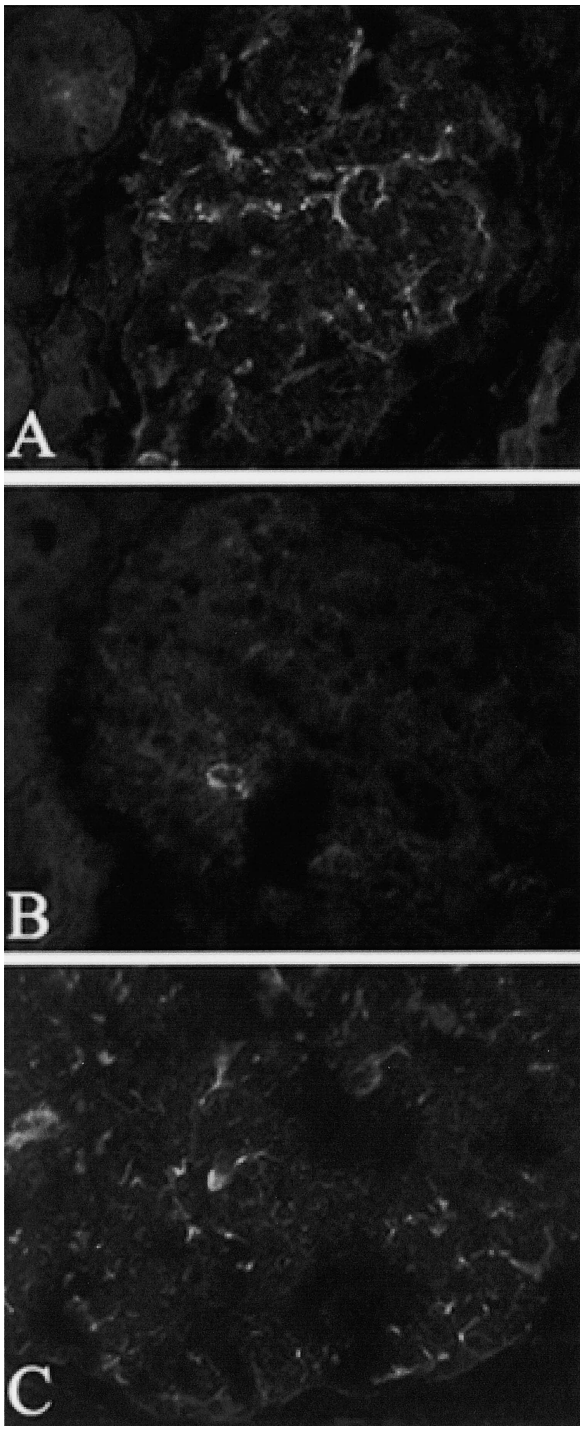

Fig. 6. Focal segmental sclerosis displaying a glomerulus positive for clusterin similar to a normal kidney (*A*), while other glomeruli demonstrate a global (*B*) or segmental (*C*) decrease in staining (IF, ×400). Immunofluorescence was done with antibodies raised against clusterin peptides.

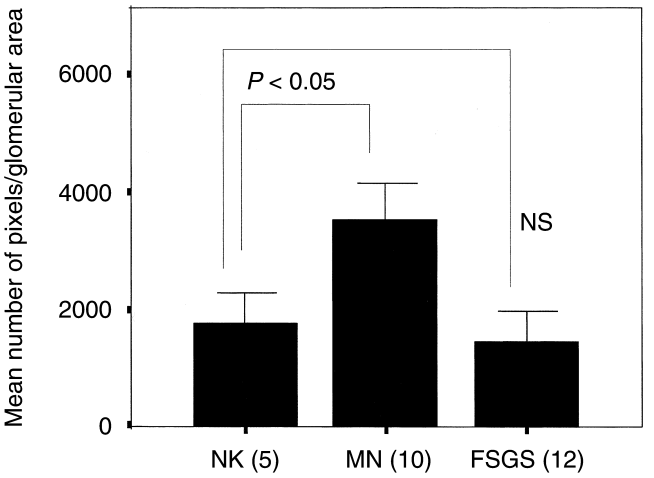

Fig. 7. Evaluation of clusterin glomerular expression in normal kidneys (NK), membranous nephropathy (MN), and focal segmental sclerosis (FSGS). Results are expressed as number of pixels/glomerular area (mean ± SD).

to proteinuria, depletion of clusterin should represent a factor aggravating the course of the disease [15, 16]. Until now, there is little, if any, information about clusterin metabolism in patients with nephrotic syndrome. The reason for this failure rests on the difficulty to measure clusterin levels in fluids. For this reason we developed a new dot-blot technique that appears to be highly reproducible and specific. Only one study has investigated the anti-inflammatory and anti-complement activity of clusterin in lupus erythematosus [21]. Another report examined clusterin levels in alcohol cirrhosis [22], the liver being the major site for its synthesis. In both cases, serum clusterin levels were low and were significantly correlated with the disease features.

This study demonstrates that serum clusterin is remarkably low in patients with nephrotic syndrome, which includes MN, FSGS and idiopathic NS, than in a control population composed of age-matched subjects. Urinary excretion also is lower in patients than in normal controls, with a trend to be higher in those who present with remission after specific treatments. Finally, the composition of clusterin is the same in serum and urine, indicating that renal handling of clusterin is not selective and is a direct function of the serum levels. In the attempt to define the mechanism responsible for depletion of clusterin in nephrotic syndrome, we looked at several putative factors, including urinary loss, loss of regulatory substances and plasma lipids. The first possibility can be reasonably ruled out since the concentration of clusterin in urine was low in the advanced stage of NS and the trend for patients showing remittance was for a restoration of normal levels.

Instead, a high statistical correlation was found by plotting the serum levels of clusterin with cholesterol, suggesting a mechanism for low clusterin to be linked with hyperlipidemia. The association between hyperlipidemia and nephrotic syndrome has been recognized for

several decades. The classic finding is increased plasma cholesterol, low- (LDL) and very low-density lipoprotein (VLDL) cholesterol, while high-density lipoproteins are unchanged (HDL1) or slightly decreased (HDL2) [23–25]. These changes derive from a combination of increased production, impaired lipolysis, and reduced receptor-mediated clearance of lipoproteins, while low HDL also can be influenced by increased urinary loss. The data presented here suggest that other regulatory mechanisms have combined effects on different components such as cholesterol and clusterin, eventually resulting in low clusterin and high cholesterol levels. It is noteworthy that lipid-lowering therapy with probucol and low-density lipoprotein apheresis ameliorates proteinuria and reduces glomerular injuries in MN and FSGS [26, 27]. We speculate that clusterin provides the logical link between hypercholesterolemia and a poor outcome of glomerulopathy.

The expression of clusterin in both normal and affected kidneys has received limited attention in the past and has been confined to experimental models of nephrosis [28, 29] cystic and dysplastic diseases. In 1994, Dvergstan et al found no clusterin staining in normal renal tissue while there was an increased expression in epithelial cells lining cysts and in some immature dysplastic tubules [28]. Our data indicate that clusterin localizes in normal glomeruli. We found an increased glomerular expression of clusterin in MN patients, whereas in sclerotic areas typical of the FSGS kidney, which are the prominent sites for the accumulation of other lipids such as LDL, it was absent. This point is pertinent to the evolution of renal lesions, since it indicates that the mechanisms regulating the glomerular expression of clusterin is unrelated to the serum levels. Indeed, Yamada et al have recently demonstrated that the mesangial expression of clusterin is up-regulated during the course of immune-mediated renal injures, this representing a critical step in protecting the kidney from complement attacks [30].

Therefore, in general it is possible that the glomerular expression of clusterin is dependent on the serum pool, this determining a defect in glomerular clusterin in FSGS. In particular cases, such as in MN, other immunolinked regulatory mechanisms could stimulate mesangial cells to produce clusterin and restore the glomerular pool of the protein. How this different regulation influences the clinical outcome in MN and in FSGS patients is currently unknown and clinical trials should focus on this aspect. The normalization of serum levels of clusterin (and indirectly of cholesterol) represents a primary objective of any therapeutic approach to both diseases.

In conclusion, these results indicate that serum and urinary clusterin is low in proteinuric patients with MN, FSGS and idiopathic NS, but the urinary losses do not account for this reduction and hypercholesterolemia appears to be the unifying feature. Glomerular clusterin is variably expressed in FSGS and in MN, suggesting a complex regulatory mechanism. According to these experimental studies, low levels of clusterin in serum should represent a cause of an unfavorable outcome.

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