

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Debiec H, Lefeu F, Kemper MJ, et al. Early-childhood membranous nephropathy due to cationic bovine serum albumin. *N Engl J Med* 2011;364:2101-10.

SUPPLEMENTARY APPENDIX

DETAILED METHODS

ASSAY FOR ANTI-BSA IGG ANTIBODIES

BSA (Sigma, St Louis, Missouri USA) was coated at a concentration of 5 µg/ml in 0.1M carbonate buffer (pH 9.6). Residual binding sites were saturated by incubation in blocking buffer for 1 h at room temperature. One hundred µl of sera diluted 1:100 in blocking buffer were added to each well. Alkaline phosphatase-conjugated anti-human IgG (Sigma) was added for detection of human antibodies and alkaline phosphatase yellow liquid substrate system for ELISA (Sigma) was used for revelation.

CIRCULATING IMMUNE COMPLEXES

Using C1q- and C3d-circulating immune complex EIA kits, sera were analyzed for immune complexes containing C1q, and C3d, implicating the classical and the common complement pathways respectively (Quidel Co. San Diego, CA, USA)

PEPTIDES

A panel of selected peptides with a spacer sequence GSGS, a biotin residue at the N-terminus and a carboxamide at the C-terminus was purchased from Mimotopes (Mimotopes. The peptide company, Melbourne, Australia). The peptides were more than 85% pure, dissolved in DMF to a concentration of 10mM, and stored at -80°C until use.

PEPTIDE BASED ELISA ASSAY

The peptide solutions were diluted in TBS-tween 0.05% and aliquots (100µl) were covalently immobilized in the wells of Reacti-Bind NeutrAvidin Coated plates (Pierce, Thermo Scientific). After blocking with Super Block blocking buffer (Pierce, Thermo Scientific, Rockford, USA) 100-fold diluted sera were applied for assays. IgG antibodies to peptides were detected using alkaline phosphatase conjugated anti-human IgG (Sigma) and revealed with alkaline phosphatase yellow liquid substrate system (Sigma).

DETECTION OF CIRCULATING BSA

BSA was detected in patients' sera by using the Bovine Albumin ELISA kit (Alpha diagnostic Intl. San Antonio, Texas, USA) based on the binding of BSA in sample to two antibodies, one immobilized on the microtiter wells, and the other conjugated to HRP.

PROTEIN ELECTROPHORESIS, IMMUNOBLOT, AND ELUTION OF IGG

BSA and human serum albumin (HSA) were electrophoresed under nonreducing conditions and transferred to PVDF membranes, according to standard protocols. Detection antibodies were peroxidase-conjugated goat anti-human antibody (Chemicon, Millipore, Billerica, MA, USA). Immunoreactive proteins were visualized with SuperSignal West Pico Chemiluminescent substrate (Pierce). IgG subclasses were identified by mouse monoclonal anti-human IgG1, IgG2, IgG3, and IgG4 antibodies, respectively (commercially provided by Margaret Goodall, University of Birmingham, UK), followed by peroxidase-conjugated sheep anti-mouse IgG (GE Healthcare, Piscataway, NJ, USA). Immunoglobulins were acid-eluted from the cores of kidney biopsy specimens from patients with membranous nephropathy. Specimens were sliced and collected by centrifugation. The sections were washed several times with PBS. Two 40-min incubations with 0.25 mM citrate buffer pH 2.5 were done. Eluted immunoglobulins were collected after centrifugation and neutralized with a 2 M NaOH and 2 M Tris, pH 7.3. The eluted IgG was used to immunoblot the BSA directly.

PURIFICATION AND CHARACTERIZATION OF CIRCULATING BSA

BSA was immunopurified from patient's serum samples by using affinity chromatography. Anti-bovine albumin agarose (Sigma) was incubated with patients' sera, control sera, or bovine serum overnight at 4°C. The resin was washed, and the bound proteins were eluted with sample buffer. The identity of immunopurified BSA was confirmed using Western blot analysis with specific chicken HRP conjugated anti-BSA antibodies (GeneTex, Inc, Irvine, CA, USA). The eluted BSA from agarose resin was also analyzed by two-dimensional electrophoresis. Cleaned protein samples were incubated with 2D sample buffer (7 M urea, 2 M thiourea, 2% Chaps, 2% Bio-Lyte pH 3-10, 60 mM DTT), the first dimension of the 2D gel was run on IPG ready strips pH 3-10 (BioRad, Hercules, CA, USA), and the second dimension was run on 8% SDS-PAGE. The separated proteins were blotted on PVDF membrane and the position of BSA was determined with chicken HRP conjugated anti-BSA antibodies (GeneTex).

IMMUNOHISTOLOGICAL ANALYSIS

We analyzed cryosections or paraffin embedded sections of normal human kidney and biopsy specimens from the patients with membranous nephropathy and with other glomerular diseases as: lupus membranous nephropathy, membranoproliferative glomerulonephritis, focal and segmental glomerulonephritis, minimal changes disease, and glomerular disease with monoclonal immunoglobulin deposits. We processed the renal biopsy specimens according to standard techniques. We detected BSA in cryosections by using rabbit polyclonal anti-BSA antibody (Invitrogen, Molecular probes, Eugene, Oregon, USA) and PLA2R in paraffin embedded sections with rabbit polyclonal anti-PLA2R antibody (Atlas antibodies AB, Stockholm Sweden) followed by goat Alexa488-conjugated anti-rabbit Fab IgG antibody (Molecular Probes). We detected C5b-9

in cryosections by using mouse monoclonal anti-human C5b-9 antibody (Dako, Glostrup, Denmark) followed by goat Alexa488 conjugated anti-mouse antibody (Molecular Probes). To analyze the composition of the glomerular immune deposits by confocal microscopy, cryosections of the biopsy specimen were first incubated with rabbit polyclonal anti-BSA antibodies (Invitrogen), then with goat Alexa488-conjugated anti-rabbit Fab IgG antibodies and goat Alexa568-conjugated anti-human IgG (Molecular probes). We examined the distribution patterns of glomerular IgG subclass deposits in membranous nephropathy patients by immunofluorescence study. The cryosections were stained with mouse monoclonal anti-human IgG1, IgG2, IgG3, and IgG4 antibodies followed by rabbit Alexa 488 conjugated anti-mouse antibodies. After being washed, sections were examined under a confocal microscope Leica TCS-SP2 and analyzed with Leica Confocal Software version 2.61.

Table 1. Baseline characteristics of the patients with membranous nephropathy

	N°	Age at diagnosis	Sex	Proteinuria g/24h	Serum creatinine $\mu\text{mol/l}$
Children	1	0.8	M	6.3	48.0
	2	0.4	F	3.0	44.0
	3	0.5	F	0.08*	17.7
	4	2.3	M	9.45	30.0
	5	3.0	M	5.7	45.0
	6	7.0	M	12.5	58.0
	7	13.0	F	25.0	60.0
	8	14.0	M	3.5	61.0
	9	15.0	M	3.1	59.0
Adults	1	73.0	M	3.0	na
	2	40.0	F	4.7	68.0
	3	46.0	F	11.56	74.2
	4	20.0	M	7.6	97.2
	5	49.0	F	6.5	80.0
	6	27.0	F	0.7	147.6
	7	58.0	M	8.3	123.7
	8	57.0	F	2.8	61.0
	9	26.0	F	8.5	57.0
	10	67.0	M	9.0	99.0
	11	32.0	F	4.5	61.0
	12	56.0	M	3.0	107
	13	19.0	M	16.9	89.0
	14	65.0	M	3.0	80.0
	15	61.0	F	8.8	63.0
	16	31.0	M	8.0	80.0
	17	60.0	M	3.0	136.0
	18	27.0	M	26.5	183.0
	19	51.0	M	5.8	na
	20	51.0	M	6.79	98.0
	21	77.0	F	3.5	150.0
	22	24.0	M	16.0	160.0
	23	56.0	F	12.0	400.0
	24	71.0	M	3.25	116.6
	25	72.0	M	7.48	130.8
	26	58.0	M	3.52	88.4
	27	65.0	M	19.14	106.9
	28	49.0	F	8.42	88.4
	29	68.0	F	2.95	144.9
	30	54.0	M	45.94	189.1
	31	35.0	F	4.3	69.8
32	31.0	F	3.57	68.0	
33	49.0	M	6.34	97.2	
34	59.0	M	5.98	99.0	
35	45.0	M	3.37	90.1	
36	43.0	M	5.6	80.0	
37	44.0	F	3.0	97.0	
38	60.0	M	12.5	122.0	
39	63.0	M	4.4	87.0	
40	43.0	M	11.2	93.0	
41	67.0	M	15.0	134.0	

*Under steroid treatment; na, not available

Table 2 : Summary of all disease controls and controls whose sera were used in experiments

	DISEASE CONTROL						CONTROL			
	N	Patient No	Age	Gender	Proteinuria g/L	Disease	N	Patient No	Age	Gender
< 5 years	N = 24	1	3.9	F	16.3	INS	N = 41	1	3.4	F
		2	3.4	M	1.3	INS		2	3.5	F
		3	4.1	F	3.1	INS		3	3.1	F
		4	1.7	F	5.8	INS		4	4.9	F
		5	3.0	M	na	INS		5	1.1	M
		6	1.3	M	11.3	INS		6	4.5	M
		7	1.7	M	na	INS		7	4.4	F
		8	2.5	M	5.0	INS		8	1.7	F
		9	3.6	F	19.4	INS		9	1.4	M
		10	4.5	M	4.1	INS		10	1.3	M
		11	2.4	F	6.2	INS		11	2.9	F
		12	2.7	M	na	INS		12	3.0	M
		13	4.0	F	23.3	INS		13	1.7	M
		14	4.7	M	0.9	INS		14	2.2	M
		15	1.6	M	9.6	INS		15	2.0	M
		16	4.7	F	2.8	INS		16	3.8	M
		17	3.4	M	10.0	INS		17	2.3	F
		18	3.0	M	38.1	INS		18	2.0	M
		19	2.2	F	na	INS		19	2.1	M
		20	3.9	M	4.7	INS		20	2.5	F
		21	3.2	F	22.0	INS		21	1.0	M
		22	3.9	F	4.0	INS		22	1.9	F
		23	3.0	M	10.8	INS		23	3.3	M
		24	4.0	M	19.9	AGN		24	3.4	M
						25	2.7	M		
						26	2.9	M		
						27	4.3	F		
						28	4.3	F		
						29	1.5	M		
						30	4.5	F		
						31	4.9	M		
						32	1.5	F		
						33	1.2	F		
						34	4.8	M		
						35	0.7	M		
						36	3.1	F		
						37	0.8	M		
						38	4.8	M		
						39	0.6	M		
						40	0.6	M		
						41	0.3	F		
5-16 years	N = 22	25	5.5	M	57.0	AGN	N = 39	42	10.1	M
		26	5.3	F	13.3	INS		43	8.8	M
		27	6.9	F	41.0	INS		44	7.6	M
		28	5.7	M	10.0	INS		45	13.3	F
		29	14.0	M	2.2	INS		46	14.5	F
		30	7.5	M	18.4	INS		47	15.8	F
		31	10.5	F	3.3	INS		48	8.2	F
		32	9.2	M	14.6	INS		49	7.4	M
		33	6.4	F	33.0	INS		50	6.6	F
		34	9.4	M	2.9	INS		51	6.2	M
		35	5.1	M	11.0	INS		52	5.4	F
36	9.8	M	2.9	INS	53	14.4	F			

	N	Patient No	Age	Gender	Proteinuria	Disease	N	Patient No	Age	Gender	
5 – 16 years	N = 22	37	8.9	M	5.9	INS	N = 39	54	14.3	F	
		38	6.6	F	11.5	INS		55	5.7	M	
		39	7.6	M	3.5	INS		56	7.2	M	
		40	5.2	M	3.0	INS		57	13.2	M	
		41	7.3	M	9.7	INS		58	10.2	M	
		42	6.6	M	9.4	INS		59	7.5	F	
		43	8.7	F	11.8	INS		60	10.5	F	
		44	9.6	M	9.8	INS		61	8.8	F	
		45	14.0	F	12.5	INS		62	10.2	F	
		46	10.0	F	4.9	INS		63	5.1	F	
									64	7.0	F
									65	7.6	M
									66	11.1	M
									67	9.4	F
									68	9.5	M
									69	5.9	M
									70	9.1	M
									71	5.4	M
									72	6.4	M
									73	5.7	M
									74	10.1	M
									75	8.4	M
						76	6.0	M			
						77	5.7	M			
						78	5.8	M			
						79	7.2	F			
						80	11.0	M			
Adult	N = 17	47	43.0	M	0.5	IgA	N = 29	81	29.0	F	
		48	37.0	M	1.5 g/24 h	IgA		82	28.0	F	
		49	70.0	M	3.0	IgA		83	27.0	F	
		50	42.0	F	3.4	IgA		84	28.0	F	
		51	42.0	M	7.0	IgA		85	40.0	F	
		52	42.0	F	0.4g/24	IgA		86	50.0	F	
		53	71.0	M	14.5	MCD		87	45.0	M	
		54	42.0	M	0.7 g/24 h	MCD		88	30.0	M	
		55	34.0	F	2 g/24 h	MCD		89	40.0	M	
		56	44.0	M	na	MCD		90	58.0	M	
		57	29.0	M	0.9	MCD		91	32.0	F	
		58	48.0	F	3.9	MCD		92	34.0	M	
		59	56.0	M	na	MCD		93	30.0	F	
		60	43.0	F	10 g/24 h	MCD		94	36.0	M	
		61	43.0	F	8.9 g/24 h	MCD		95	45.0	M	
		62	30.0	M	6 g/24 h	MCD		96	40.0	F	
		63	51.0	F	4.7 g/24 h	MPGN		97	23.0	F	
									98	26.0	M
									99	19.0	M
									100	57.0	M
									101	54.0	F
									102	68.0	F
						103	72.0	M			
						104	31.0	M			
						105	36.0	M			
						106	43.0	M			
						107	38.0	F			
						108	55.0	M			
						109	60.0	F			

INS, idiopathic nephrotic syndrome ; AGN, acute glomerulonephritis ; IgA, IgA nephropathy ; MCD, minimal change disease ; MPGN, membranoproliferative GN. In children, the term « INS » was used instead of MCD because most children did not undergo a kidney biopsy. Children controls were sampled in hospitals where they were referred for various health problems. Non of them had glomerular disease. Adult controls were healthy subjects. Proteinuria was expressed as g/L or g/24h.

Table 3 : Amino acid sequence comparison of synthesized peptides derived from BSA with the corresponding sequence in HSA

N°	BSA/ HSA
1	<u>D E S H A G C E</u> K S L H E S A E N C D K S
2	<u>D D S P D L P K L K P D P N T L</u> C D D N P N L P R L V R P E V D V M C
3	<u>D P N T L C D E F K A D E</u> E V D V M C T A F H D N E
4	<u>C D E F K A D E K K F W G K Y</u> C T A F H D N E E T F L K K Y
5	<u>D E K K F W G K Y L E T A</u> D N E E T F L K K Y L Y
6	<u>E L L Y A N K Y N G V Q</u> E L L F F A K R Y K A A F T E C
7	<u>P K I E T M R E K V L T S S</u> P K L D E L R D E G K A S S
8	<u>E K D A I P E D L P P L T A D F A E D K</u> E N D E M P A D L P S L A A D F V E S K
9	<u>H P E Y A V S V L L</u> H P D Y S V V L L L
10	<u>P H A C Y T S V F D K L K H L V D E P</u> P H E C Y A K V F D E F K P L M E E P
11	<u>V G T R C C T K P E S E R M</u> V G S K C C K H P E A K R M
12	<u>L S L I L N R L C</u> L S V V L N Q L C
13	<u>P K A F D E K L F T</u> P K E F N A E T F T
14	<u>T L P D T E</u> Q I T L S E K E R Q I

Table 4 : Summary of serum and biopsy analysis in patients

	Patient N°	Age	Circulating BSA	Anti-BSA antibody subclasses in serum		BSA in biopsy	PLA ₂ R in biopsy
				IgG1	IgG4		
Children	1	0.8	cBSA	+	++	na	na
	2	0.4	cBSA	+	++	na	-
	3	0.5	cBSA	+	++	+	-
	4	2.3	cBSA	+	++	+	-
	5	3.0	-	-	-	-	-
	6	7.0	-	-	-	na	na
	7	13.0	-	-	-	na	na
	8	14.0	-	-	-	-	-
	9	15.0	-	-	-	na	na
Adults	1	73.0	nBSA	++	+	-	-
	2	40.0	nBSA	+	++	-	+
	3	46.0	nBSA	+	++	-	+
	4	20.0	nBSA	+	++	-	+
	5	49.0	low nBSA	+	++	-	+
	6	27.0	-	+	++	na	na
	7	58.0	-	++	+	na	na
	8	57.0	-	-	-	-	-
	9	26.0	-	-	-	-	-
	10	67.0	-	-	-	-	-
	11	32.0	-	-	-	-	+
	12	56.0	-	-	-	-	+
	13	19.0	-	-	-	-	+
	14	65.0	-	-	-	-	+
	15	61.0	-	-	-	-	+
	16	31.0	-	-	-	-	+
	17	60.0	-	-	-	-	+
	18	27.0	-	-	-	-	+
	19	51.0	-	-	-	-	+
	20	51.0	-	-	-	-	+

Table 5 : Diet characteristics and clinical setting at onset of nephrotic syndrome (NS) in the 4 patients with cBSA-related membranous nephropathy

Patient N°	Diet			Signs at onset of NS
	<i>Breast feeding</i>	<i>Cow's milk formula*</i>	<i>Diary product after 12 months</i>	
1	Yes	Formula not specified	Yes	None
2	Yes	Modilac 1 st age, then 2 nd age until 12 months	Yes	Diarrhea
3	Yes	Beba HA, Beba 1, then Beba 2 until 12 months	Yes	None
4	Yes	Formula not specified	Yes	Neutropenia, anemia, thrombocytosis (transient)

* No change in diet at time of first diagnosis

Table 6: Pathologic characteristics of the 4 children with cBSA-related membranous nephropathy

Patient N°	Light microscopy				Immunofluorescence		Electron microscopy
	<i>Stage</i>	<i>Mesangial proliferation</i>	<i>Glomerular sclerosis</i>	<i>Interstitial sclerosis</i>	<i>Immune components</i>	<i>IgG subclass</i>	<i>Deposits</i>
1	II	Yes	No	No	IgG, C3	NA	Subepithelial
2	I	Very mild	No	No	IgG, C3 (IgA, IgM)*	NA	NA
3	II-III	Very mild	No	No	IgG, C3 (IgA, IgM Mes.)*	IgG1, IgG4	Subepithelial (mesangial, rare)
4	I-II	No	No	No	IgG, C3 (+ rare IgG deposits in TBM)*	IgG1, IgG4	NA

* indicates weak deposits

Mes., mesangium ; TBM, tubular basement membrane; NA, not available

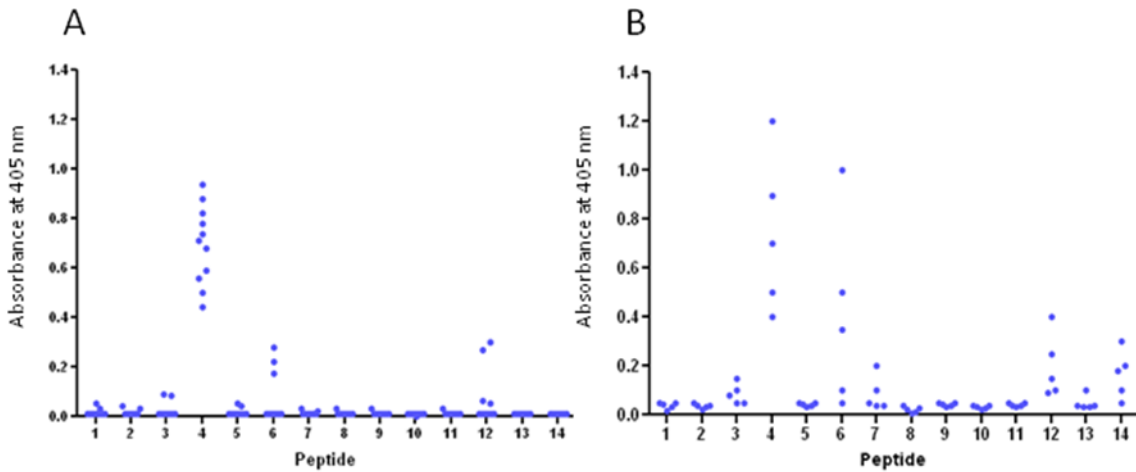
Atypical features were seen in Pt1 (mesangial proliferation) and Pt4 (rare IgG deposits along TBM). C5b-9 was detected in the 2 available biopsies (Pt3 and Pt4).

Table 7 : Treatment and outcome of the 4 children with cBSA-related membranous nephropathy

Patient N°	Treatment	Time to remission	Relapse	Treatment of relapse	Last follow-up
1	Prednisone, then MMF and ARB	Progressive incomplete remission	No	-	3y, 6 mo PR
2	Ciclosporine (15 mo)	10 mo	Yes 4 y, 3 mo	Ciclosporine, Prednisone	5 y, 7 mo CR
3	Prednisone (12 mo)	3 mo	No	-	6 y CR
4	Prednisone (4.5 mo)	3 mo	Yes 4 y, 6 mo	Prednisone	8 y CR

MMF, mycophenolate mofetil ; ARB, Angiotensin II receptor blocker ; PR, Partial remission ; CR, Complete remission

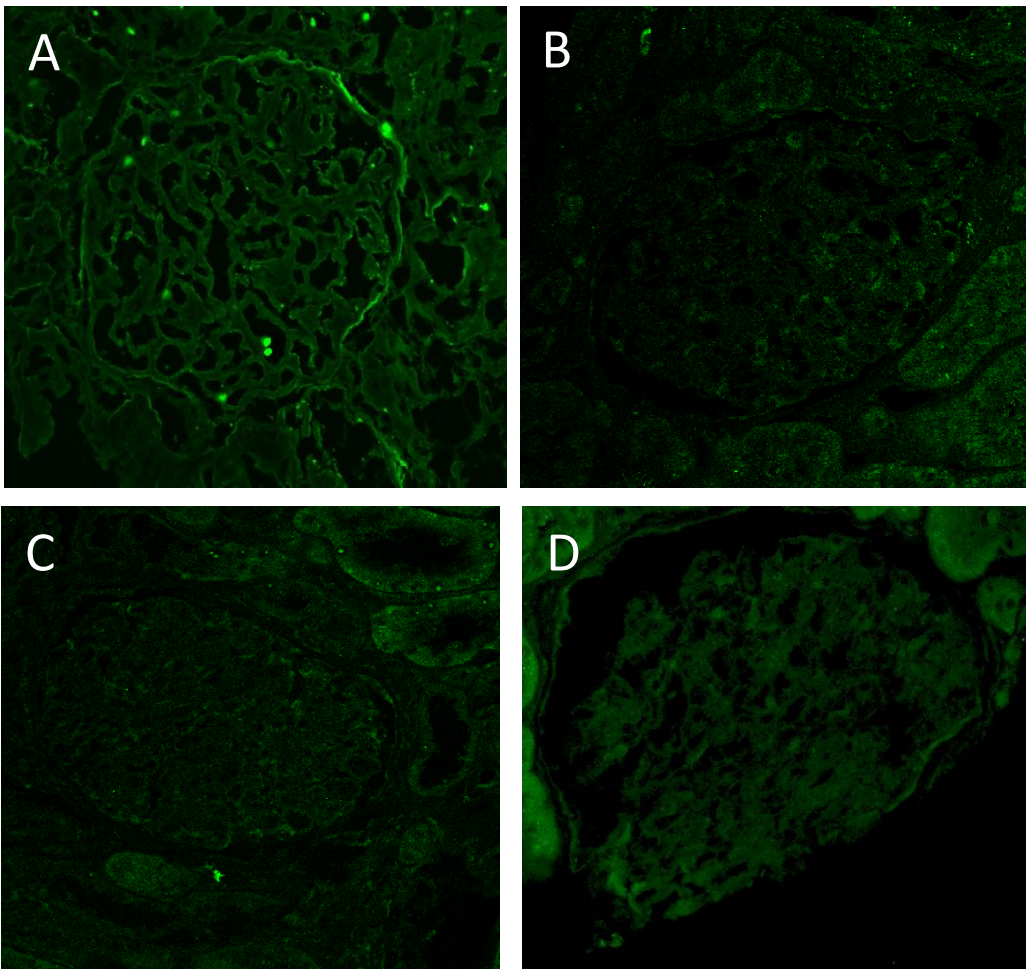
Figure 1 Supplement. **Epitope mapping.**



Panels A and B show IgG-binding abilities of synthetic peptides. The synthesized peptides were covalently immobilized on the wells of immunoassay plates and applied to ELISA using diluted serum from patients with membranous nephropathy (D, N=11) or healthy control patients (E, N=5). Individual patients are represented by circles.

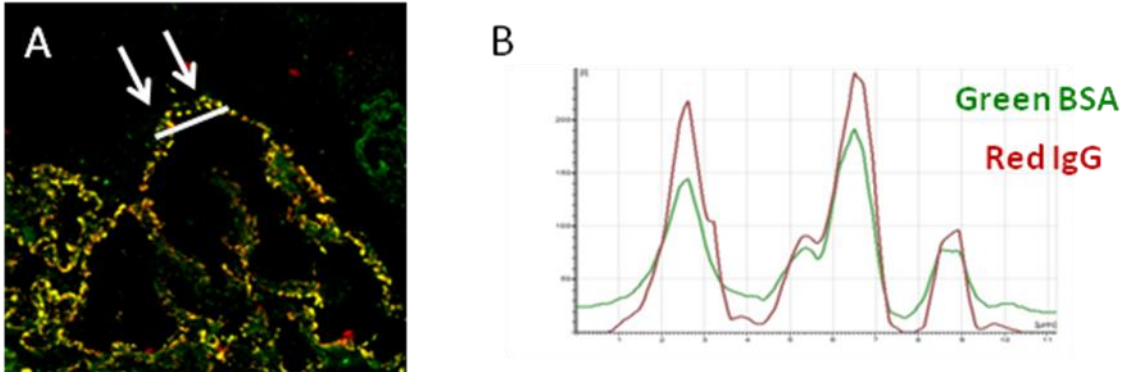
Panel C shows amino-acids 147-161 of BSA peptide 4, with the corresponding sequence in HSA. Underlined residues represent the two linear epitopes that are BSA specific.

Figure 2 Supplement **Expression of BSA in biopsy specimen**



Panels A to D show staining for BSA in biopsy of patient with lupus membranous nephropathy (A), focal and segmental glomerulonephritis (B), minimal change disease (C), and glomerular disease with monoclonal immunoglobulin deposition (D).

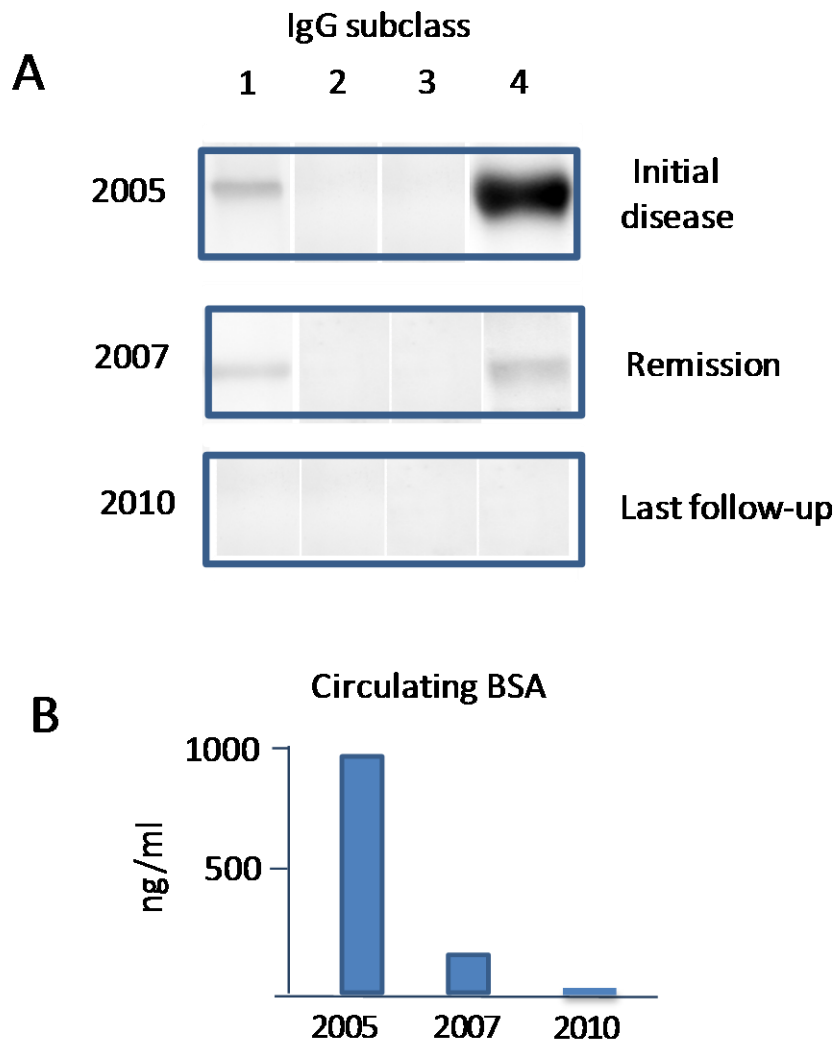
Figure 3 Supplement **Colocalization of BSA and IgG in immune deposits: quantitative analysis**



Panel A is high magnification images of deposit in glomerular capillary loop from the glomerulus that have been double-labeled with rabbit polyclonal anti-BSA antibody (green) and anti-human IgG antibodies (red). Panel A shows a merged image

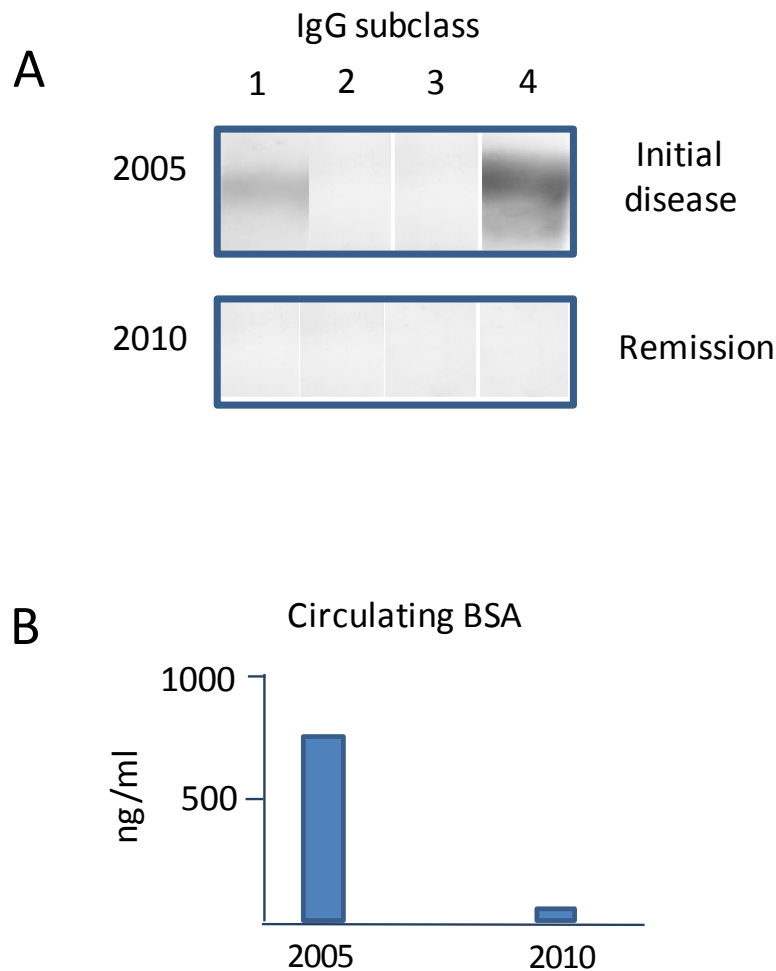
Panel B shows a quantitative analysis of the fluorescence recorded across sections of a representative capillary loop (bar). Note colocalization of the two signals, which indicates that subepithelial immune deposits are composed of the BSA antigen (green) and of IgG (red).

Figure 4 Supplement Anti-BSA antibodies, circulating BSA and disease activity in a patient with membranous nephropathy



Serum samples were collected from patient #3. The patient is a 6-month -old Caucasian female who was diagnosed with membranous nephropathy (stage II-III). She was treated with prednisone (2x10 mg). Proteinuria was normalized after 3 months of treatment, then steroids were tapered and discontinued after 12 months. At last follow-up in November 2010 proteinuria was in normal range with normal renal function.

Figure 5 Supplement **Anti-BSA antibodies, circulating BSA and disease activity in a patient with membranous nephropathy**



Serum samples were collected from patient#2. The patient is a 5-months-old Caucasian female who was diagnosed in september 2005 with membranous nephropathy (Stage 1) responsible for nephrotic syndrome. She was induced in remission with ciclosporine which was stopped after 15 monts. A relapse occurred 2.5 years after the treatment had been stopped. No serum was available at relapse. Remission was obtained with ciclosporine and steroids.