**Supplementary material**

**Morpho-Functional Effects of C5 Convertase Blockade in**

**Immune complex-Mediated Membranoproliferative Glomerulonephritis:**

***Report of two cases with Evidence of Terminal Complement Activation***

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**EAGLE Study Organization** *(number of included patients in brackets):*

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*Centres including patients* – Unit of Nephrology and Dialysis, ASST Papa Giovanni XXIII, Bergamo (G. Remuzzi, P. Ruggenenti, E. Mondo, S. Rota, C. Carrara, V. Portalupi; n=5); Unit of Nephrology and Pediatric Dialysis, Policlinico Sant’Orsola-Malpighi, Bologna (A. Pasini, G. Monitini, E. Monti) and Unit of Nephrology and Dialysis Ospedale degli Infermi di Rimini, Rimini (A. Rigotti, F. De Giovanni, n=1); Unit of Nephrology and Dialysis, Ospedale di Bolzano, Bolzano (B. Giacon, R. M. Lerchner, W. Passler, n=1); Unit of Nephrology and Dialysis, Policlinico “G. Martino”, Messina (D. Santoro, L. Visconti, n=1); Cattedra di Nefrologia, Università Federico II, Napoli (A. Pisani, E. Riccio, n=1); Unit of Nephrology Ospedale Cà Foncello, Treviso (A. Pasi, M. Dugo, C. Tuono, n=1);

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*Activities of the Clinical Research Centre –* Patient recruitment and care, data recording (B. Ruggiero, E. Daina, E. Bresin, S. Gamba, S. Prandini, V. Lecchi, D. Cugini, G. Gherardi); Monitoring, Drug Distribution and Pharmacovigilance (N. Rubis, O. Diadei, A. Villa, D. Villa); Regulatory Affairs (P. Boccardo, S. Peracchi); Database and Data Validation (D. Martinetti), Data Analysis (A. Perna, F. Peraro, G.A. Giuliano), Centralized Laboratory Measurements (F. Gaspari, F. Carrara, S.Ferrari, N. Stucchi, A. Cannata), *Complement Activity Tests* (M. Noris, S. Bettoni, M. Alberti, P. Cuccarolo); Light and electron microscopy and immunofluorescence (C. Carrara, M. Abbate, P. Rizzo, G. F. Marchetti, A. Sonzogni)

***Kidney biopsy evaluation***

Tissue samples from baseline and post-treatment kidney biopsies were processed at the Department of Pathological Anatomy of the Azienda Socio-Sanitaria Territoriale Papa Giovanni XXIII (Bergamo, Italy) using standard techniques for light microscopy, immunofluorescence and, electron microscopy. The processing for electron microscopy was completed at the Mario Negri Institute for Pharmacological Research IRCCS (Bergamo, Italy). Morphological findings were evaluated by two renal pathologists who were not blinded to whether biopsies were pre-treatment or post-treatment.

For light microscopy, Duboscq-Brazil-fixed, paraffin-embedded kidney biopsy tissue was cut at 3-μm thickness, deparaffinised and stained with hematoxylin and eosin, periodic acid-Schiff reagent and Masson’s trichrome. Standard immunofluorescence was performed on 2-μm cryostat sections using polyclonal FITC-conjugated antibodies to IgG, IgM, IgA, C3c, C1q, and kappa and lambda light chains (DakoCytomation, Glostrup, Denmark). For C3 and C5b-9 co-staining and for staining quantification technique see below. For ultrastructural analysis, biopsy specimens were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 4 hours at 4°C, and then washed in cacodylate buffer. Kidney fragments were then postfixed in 1% osmium tetroxide for 1 hour, dehydrated through ascending grades of alcohol, and embedded in Epon resin (Electron Microscopy Science, Hatfield, PA). Ultrathin sections (100nm) were cut on an EM UC7 ultramicrotome (Leica Microsystems, Mannheim, Germany), stained with uranyl acetate and lead citrate, and examined with transmission electron microscope (Morgagni 268D, Philips, Brno, Czech Republic).

***Double immunofluorescence staining for C3 and C5b-9 and deposit quantification***

Frozen sections were fixed in acetone for 10 min at 4°C. The sites of nonspecific binding were blocked with PBS1X/BSA1%. Sections were incubated with rabbit anti-human C5b-9 antibody (1:200, Calbiochem, Merck Millipore Ltd., Nottingham, UK) followed by Cy3-conjugated secondary antibody goat anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories), and then with FITC-conjugated C3c antibody. Negative controls were obtained by omitting the primary antibody on adjacent sections. Fluorescence was examined through an inverted confocal laser microscope (LSM510 Meta; Zeiss, Jena, Germany). C3 and C5b-9 deposits were quantified in all of the glomeruli that were found in kidney biopsy specimens, using ImageJ software (National Institutes of Health, Bethesda, MD; <http://imagej.nih.gov/ij>), and values were expressed as percentage of positive area in the total glomerular area.

***Determination of complement profile***

sC5b-9 levels were assessed with the enzyme-linked immunoassay commercially available from Quidel (MicroVue SC5b-9 Plus). Serum C3 and C4 levels were measured through kinetic nephelometry. Factor H (FH) levels and the presence of anti-FH antibodies in the serum/plasma of the patients were evaluated by Enzyme-Linked Immunosorbent Assay (ELISA) (1). C3NeF activity was tested evaluating the capacity of IgGs purified from plasma to stabilize the convertase of the alternative pathway, as previously described (2).

***GFR measurement***

Glomerular filtration rate was measured in the laboratories of the Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Aldo e Cele Daccò Clinical Research Center (Ranica, Bergamo, Italy) by using the iohexol plasma clearance technique as previously described (3). Briefly, on the morning of renal function evaluation, 5 mL of iohexol solution (Omnipaque 300, GE Healthcare, Milan, Italy) were injected intravenously over 2 minutes. Venous blood was sampled at different times and iohexol plasma levels were measured through high-performance liquid chromatography. The clearance of iohexol was calculated according to a one-compartment model (CL1) by the formula: CL1=Dose/AUC, where AUC is the area under the plasma concentration-time curve. Plasma clearances were corrected by using the CL formula =(0.9907786CL11)-(0.0012186CL12), and GFR values were normalised to 1.73 m2 of body surface area (BSA).

***Genetic analyses***

***Next Generation Sequencing***

Genomic DNA was extracted from peripheral blood by the NucleonTM BACC2 Genomic DNA extraction kit (GE Healthcare, Little Chalfont, UK). Genetic screening was performed using a next generation sequencing minipanel for simultaneous sequencing of six complement genes (complement factor H, *CFH*; complement factor I, *CFI;* membrane cofactor protein*, CD46,* complement Factor B, *CFB; C3;* and thrombomodulin*, THBD).* Sequence data were analysed using the TorrentSuite Software 3.6 and genetic variants were annotated with ANNOVAR software.

Genetic screening disclosed two *CFH* common variants in both patients (Supplementary Table 1): p.V62I (c.184G>A; rs800292), reported as protective in patients with C3G (4) and p.Y402H (c.1204C>T; rs1061170), and which have been described as being over-represented in some cohorts of DDD patients compared to controls (5, 6). Functional studies showed that both V62 and H402 affect factor H function on alternative pathway regulation (7-9).

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| --- | --- | --- | --- |
| **CFH SNP** | **Risk allele** | **Patient 1** | **Patient 2** |
| p.V62I; c.184G>A; rs800292 | V | GG | GA |
| p.Y402H, c.1204C>T; rs1061170 | H | CT | CT |

**Supplementary Table 1**. *CFH* polymorphisms identified in the two patients.

***CFH-CFHR copy number variation analysis***

Multiplex ligation-dependent probe amplification (MLPA) was performed with the SALSA MLPA P236-A3 ARMD Kit (MRC- Holland) to detect genomic rearrangements affecting *CFH*, complement factor H-related 1 (*CFHR1*), *CFHR2*, *CFHR3* and, *CFHR5*. This assay revealed only the heterozygous common deletion of *CFHR3-CFHR1* genes in *Patient 1* (10).

***Detailed pathology findings***

*Pre-treatment biopsy (Patient 1)*

Light microscopy revealed mild mesangial proliferation and diffuse endocapillary hypercellularity with marked neutrophil infiltration, conferring a vaguely lobulated appearance to the glomeruli (Figure 2). In addition, there was moderate focal tubulointerstitial inflammation. Immunofluorescence evaluation showed diffuse C3 and C5b-9 glomerular parietal deposits (3+) with a similar pattern and distribution, together with diffuse parietal IgM (2+), but negative IgG, IgA, C1q, fibrin, kappa and lambda light chain. Electron microscopy detected frequent subendothelial electron-dense deposits, accompanied by focal duplication of the GBM and occasional intramembranous band-like electron-dense deposits. The mesangium had expanded due to the accumulation of scattered electron-dense deposits, increased cellularity and matrix. Podocytes exhibited diffuse foot process effacement.

*Post-treatment biopsy (Patient 1)*

Light microscopy revealed improvements in inflammatory features, with reduced mesangial and endocapillary hypercellularity, but increased mesangial matrix, more accentuated glomerular lobulation and adhesions to the Bowman’s capsule, with an increase in segmental glomerular sclerosis from 15% at baseline to 40% at study end. Tubulointerstitial damage did not change appreciably compared to baseline. The patterns and intensity of immunofluorescence staining for C3 (3+) and IgM (2+) in the two biopsies were similar. Conversely, median (IQR) staining for C5b-9 (p=0.021) decreased significantly, from 23.6% (22.7 to 24.9%) at baseline to 18.2% (14.8 to 20.6 %) in the post-treatment biopsy (see Supplementary Material for staining quantification technique). The post-treatment biopsy was also notable due to the deposition of segmental IgG and kappa light chain (1+) in glomerular capillaries, and revealed persistent subendothelial and intramembranous deposits, some of which were prominent, ribbon-like and highly electron-dense. Mesangial deposits appeared less frequent and were occasionally more electron-dense than pretreatment. The GBM exhibited segmental double contours. Some subendothelial and mesangial deposits had a partial, punctuate, powdery texture. Occasional, scattered electron-dense deposits were identified in the glomerular subepithelial location, in the Bowman’s capsule and in the tubular basement membrane. Foot process effacement was extensive.

*Pre-treatment biopsy (Patient 2)*

Light microscopy revealed initial glomerular lobulation, segmental duplication of the GBM, moderate mesangial proliferation and exudative features, including focally severe endocapillary hypercellularity with neutrophil infiltration (Figure 2). In addition, one glomerulus exhibited tuft-to-capsule adhesion with associated segmental sclerosis. The inflammatory infiltrates observed in the interstitium were sparse. Immunofluorescence evaluation showed diffuse C3 (3+) and C5b-9 (2+) glomerular parietal deposits with a similar pattern and distribution, together with parietal IgM (2+), IgG (1-2+) and C1q (2+) and less intense kappa (1+) and lambda light chains (1+). There was no deposition of IgA and fibrin. Electron microscopy detected frequent intramembranous electron-dense deposits and focal subendothelial electron-dense deposits. The mesangium was expanded due to the accumulation of scattered electron-dense deposits, increased cellularity and matrix. Podocytes exhibited diffuse foot process effacement. Notably, electron microscopy evaluation revealed segmental features of glomerular microangiopathic injury, characterized by endothelial cell swelling with a loss of the normal fenestration and with a widening of the subendothelial space, which was occupied by electron-lucent, “fluffy” material (Figure 3).

*Post-treatment biopsy (Patient 2)*

Light microscopy revealed improvements in inflammatory features, with reduced mesangial and endocapillary hypercellularity, but more mesangial matrix, more accentuated glomerular lobulation and multiple adhesions to the Bowman’s capsule, with an increase in segmental glomerular sclerosis from 6% at baseline to 30% at study end. The sparse interstitial inflammatory infiltrates observed in the first biopsy were replaced by focal interstitial fibrosis and tubular atrophy. The intensity of IF staining for C3 did not change at repeat biopsy. Conversely, median (IQR) staining for C5b-9 significantly (p=0.019) decreased from 15.8% (15.2 to 16.5%) at baseline to 10.7% (8.5 to 15.0 %) at post-treatment biopsy (see Supplementary Material for staining quantification technique). The staining of the other antisera did not change appreciably compared to baseline, with the exception of C1q deposits, which almost disappeared (from “2+” to “traces” intensity). The post-treatment biopsy revealed stable intramembranous and subendothelial electron-dense deposits, characterised by a more electron-dense appearance compared to those seen in the previous biopsy, diffuse GBM duplication and the added finding of isolated powdery subendothelial deposits, similar to those described in *Patient 1*. There was a slight increase in the number of mesangial deposits. The degree of podocyte foot process effacement improved significantly (from a diffuse pattern of distribution to a focal pattern). Glomerular microangiopathic lesions recovered fully.

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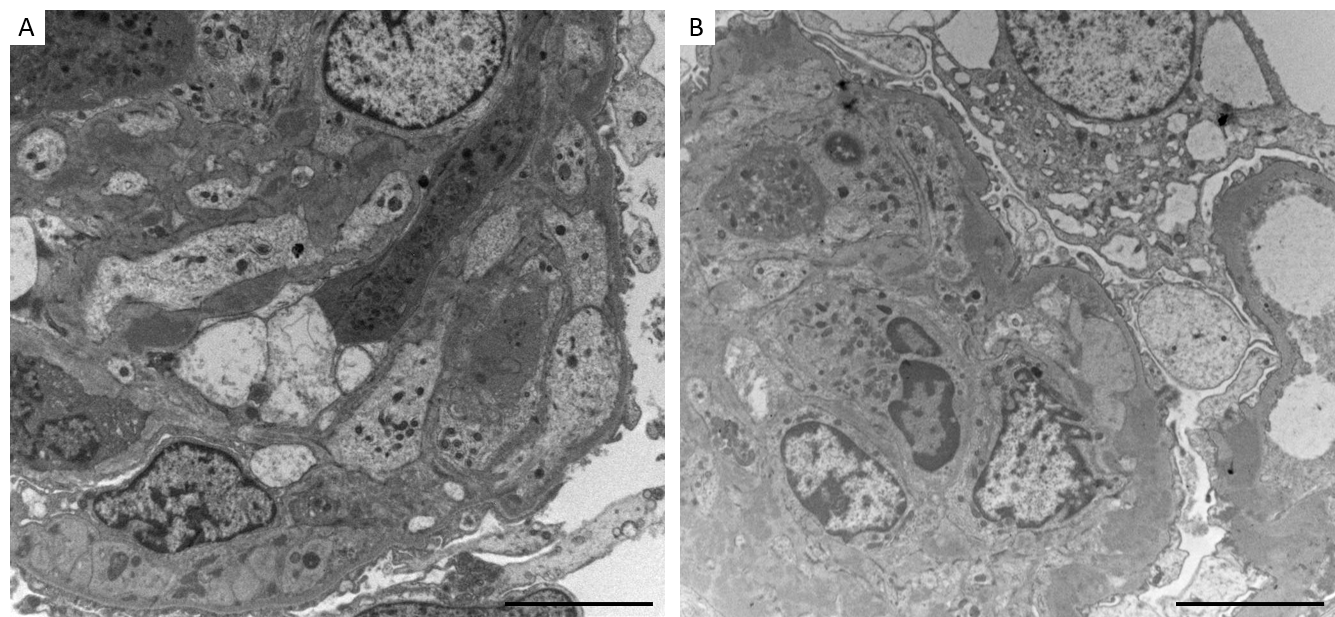
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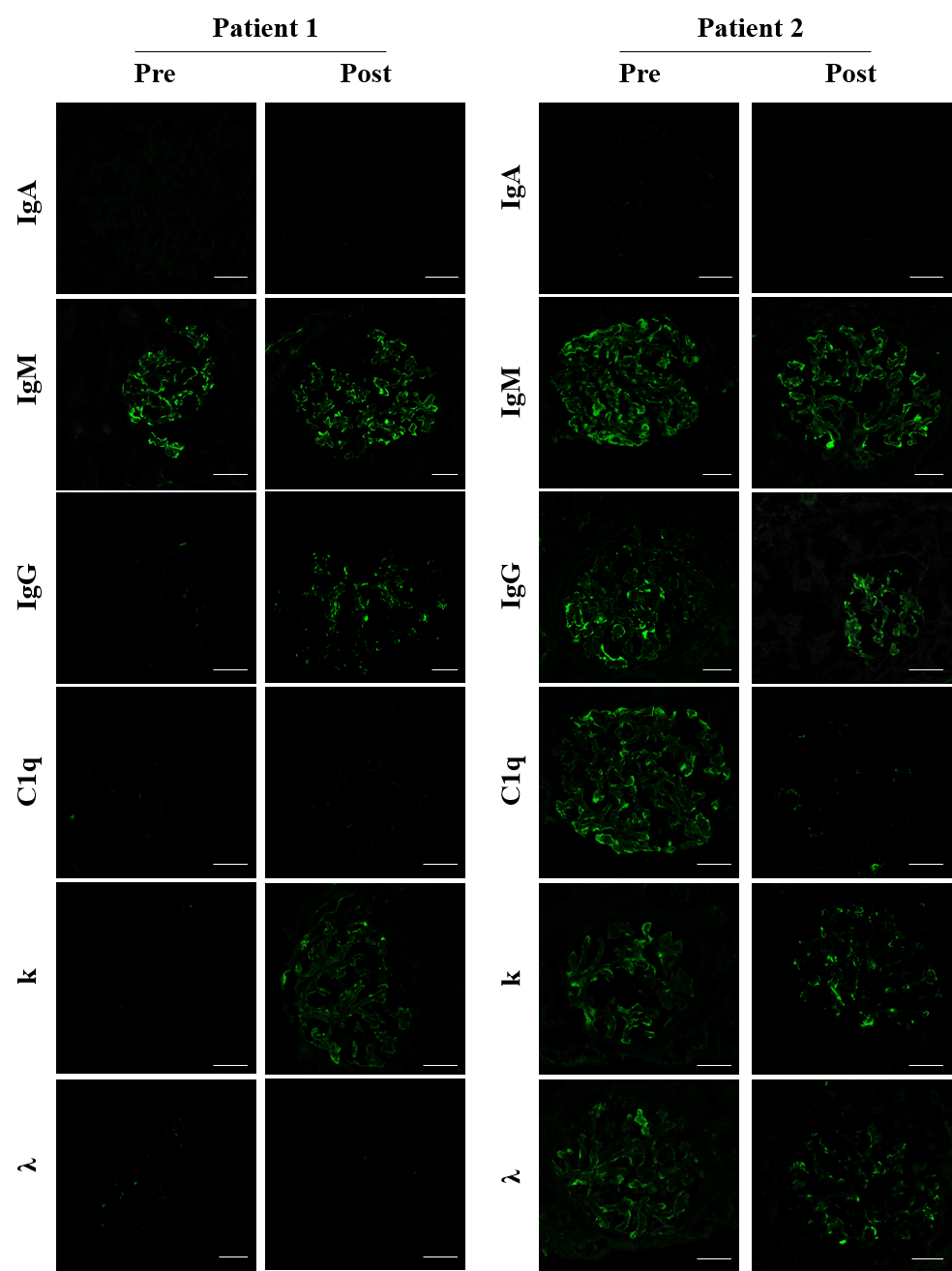
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**Supplementary Figure 1.** Electron micrographs of glomerular capillaries from pre-treatment biopsies of two additional non-responders from the EAGLE study (A: C3GN; B: IC-MPGN). Glomeruli from both specimens showed predominantly subendothelial, intramembranous and mesangial electron dense deposits and, in the patient with IC-MPGN, hump-like subepithelial electron dense deposits. Marked leukocyte accumulation was associated with membranoproliferative pattern and remodelling of the glomerular basement membrane, without typical microangiopathic changes suggestive of TMA. Scale bars 5000 nm.

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**Supplementary Figure 2.** Immunofluorescenceevaluation of pre- and post-treatment biopsies from patient 1 and patient 2. Detailed pathology description available as Supplementary Material. Scale bars 50 μm.

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