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Anti-slit diaphragm antibodies on kidney biopsy identify pediatric patients with steroid-resistant nephrotic syndrome responsive to second-line immunosuppressants

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Valentina Raglianti[1,2], Maria Lucia Angelotti[2], Luigi Cirillo[1,2], Fiammetta Ravaglia[3], Samuela Landini[4], Viviana Palazzo[4], Maria Elena Melica[2], Giulia Antonelli[1], Carolina Conte[1], Elisa Buti[1], Carmela Errichiello[1], Letizia De Chiara[2], Anna J. Peired[2], Laura Lasagni[2], Anna Maria Buccoliero[5], Marco Allinovi[6], Anna Manonelles Montero[7], Josep Maria Cruzado[7], Maurizio Bruschi[8], Gian Marco Ghiggeri[8], Andrea Angeletti[8], Hans-Joachim Anders[9], Elena Lazzeri[2], Benedetta Mazzinghi[1], Francesca Becherucci[1,2] and Paola Romagnani[2]

[1]Nephrology and Dialysis Unit, Meyer Children's Hospital IRCCS, Florence, Italy; [2]Department of Biomedical, Experimental and Clinical Sciences "Mario Serio," University of Florence, Florence, Italy; [3]Nephrology and Dialysis Unit, Santo Stefano Hospital, Prato, Italy; [4]Medical Genetics Unit, Meyer Children's Hospital IRCCS, Florence, Italy; [5]Pathology Unit, Meyer Children's Hospital IRCCS, Florence, Italy; [6]Nephrology, Dialysis and Transplantation Unit, Careggi University Hospital AUOC, Florence, Italy; [7]Renal Transplant Unit, Nephrology Department, Bellvitge University Hospital, Barcelona, Spain; [8]UO Nephrology Dialysis and Transplant, IRCCS Istituto Giannina Gaslini, Genoa, Italy; and [9]Division of Nephrology, Medizinische Klinik and Poliklinik IV, Klinikum der LMU München, Munich, Germany

Podocytopathies represent a group of glomerular disorders associated with minimal changes (MC) or focal segmental glomerulosclerosis (FSGS) lesion patterns at biopsy and heterogeneous responses to steroids. Anti-nephrin antibodies were previously found in such patients, suggesting an autoimmune form of podocytopathy. High resolution confocal microscopy on kidney biopsies of a cohort of 128 pediatric patients revealed localization of IgG along the slit diaphragm in 30% of patients with MC and 25% of those with FSGS, but not in other lesion patterns. Anti-nephrin IgG ELISA assay in the serum and stimulated emission depletion microscopy of kidney biopsies showed IgG-nephrin co-localization only in 77.8% of cases. Similar observations were obtained in a cohort of 48 adult patients with MC or FSGS at kidney biopsy, where IgG-nephrin colocalization was only 44.4%, suggesting the existence of autoantibodies binding to other slit proteins. Patients with anti-slit antibodies showed nephrotic syndrome at onset in 94.4% of cases. Patients with primary steroid-resistance had anti-slit antibodies in 27%, while those with secondary steroid-resistance in 87.5% of cases, irrespective of the histopathological lesion pattern. Steroid-resistant patients with anti-slit antibodies responded to second-line immunosuppressants in 92.3% vs. only 20% of patients that were anti-slit negative. No patient with anti-slit antibodies developed kidney failure vs. 51.7% of those negative for antibodies (66.7% with a genetic cause and 41.2% with a non-genetic cause). Thus, the detection of anti-slit antibodies can identify patients with an autoimmune podocytopathy responsive to treatment with second-line immunosuppressants, irrespective of the histopathological lesion pattern at biopsy.

Correspondence: *Paola Romagnani, Department of Biomedical, Experimental and Clinical Sciences "Mario Serio," University of Florence, Viale Pieraccini 6, 50139 Florence, Italy, and Nephrology and Dialysis Unit, Meyer Children's Hospital IRCCS, Viale Pieraccini 24, 50139 Florence, Italy. E-mail: paola.romagnani@meyer.it*

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Podocytopathies are kidney diseases in which podocyte injury causes proteinuria.[1] Children and young adults with severe nephrotic syndrome (NS) frequently respond to treatment with steroids. These patients usually show a lesion pattern of minimal changes (MCs) at kidney biopsy and a favorable long-term kidney prognosis, although prolonged immunosuppression can be a challenge.[1,2] By contrast, the 10% to 20% of patients with a steroid-resistant NS (SRNS) frequently show a focal segmental glomerular sclerosis (FSGS) lesion pattern at biopsy, a variable response to second-line immunosuppressant drug therapy and heterogeneous long-term outcome, implying a diverse etiology.[1,2] Genetic testing identifies a genetic podocytopathy or phenocopy in 30% to 60% of patients with SRNS, who are usually resistant to second-line immunosuppressant therapy and hence frequently progress to kidney failure.[1–3] Patients with nongenetic SRNS can achieve remission with second-line immunosuppressant therapy linked to a more favorable long-term outcome.[4] Nongenetic patients who are multidrug

Lay Summary

Nephrotic syndrome (NS) characterizes many patients affected by various kidney diseases associated with different lesion patterns at kidney biopsy and heterogeneous responses to steroids. Patients with steroid-resistant NS (SRNS) show variable response to second-line therapies and frequently show poor long-term prognosis. Here, we demonstrate that a subset of patients with SRNS shows antibodies against nephrin or other slit proteins that can be visualized on kidney biopsy. Patients with anti-slit antibodies show a good response to second-line immunosuppressants and a favorable long-term outcome, despite a more severe clinical presentation, in comparison to all other patients with SRNS. In conclusion, detection of anti-slit antibodies in kidney biopsies, in addition to measurement of anti-nephrin antibodies in the serum, of patients with SRNS may represent a novel tool for personalized management, by allowing identification of patients more likely to respond to intense immunosuppression.

resistant usually progress to kidney failure.[5,6] Overcoming the current trial-and-error therapeutic approach in SRNS and limiting the initiation of secondary immunosuppressive therapy to those who could benefit from it is still an unmet medical need. Recently, Watts *et al.* reported the presence of autoantibodies directed against nephrin in kidney biopsies and sera of a subset of patients with NS and a MC lesion pattern at kidney biopsy as well as of relapsing NS after kidney transplantations.[7,8] We hypothesized that identification of anti-slit antibodies on kidney biopsy could identify patients with an autoimmune podocytopathy, which, despite steroid resistance, may respond to second-line immunosuppressant.

METHODS

Patients

We screened all the 128 pediatric patients who underwent kidney biopsy at Meyer Children's Hospital IRCCS from January 2010 until December 2023, with frozen kidney biopsy still available (Supplementary Figure S1) for the evaluation of IgG positivity of the filtration slit. Immunolabeling experiments were conducted in a blinded manner by 2 independent investigators. Five age-matched healthy kidney biopsies taken from nephrectomy specimens performed for kidney tumors served as controls. Sixty-two of the 128 patients showed a podocytopathy pattern (MC or FSGS) at kidney biopsy and, among them, 45 had a SRNS clinical phenotype (Supplementary Figure S1 and Supplementary Table S1). These 45 patients with SRNS with frozen biopsy sample available were compared with all the remaining age-matched patients with SRNS followed up in the same period (n = 51; Supplementary Figure S1 and Supplementary Table S2). Patients with SRNS were genetically tested at Meyer Children's Hospital IRCCS and defined as "genetic" and "nongenetic" patients according to international standards.[3,9,10] Chronic kidney disease (CKD) and remission were defined according to Kidney Disease: Improving Global Outcomes guidelines, as previously described.[3,9,11] Treatment with renin-angiotensin system inhibitors and immunosuppressants followed the Kidney Disease: Improving Global Outcomes guidelines in terms of drug choice and dosage, as reported.[2,10,11] We also assessed a validation cohort of adult patients from Careggi University Hospital (AOUC), Florence, Italy, and Bellvitge University Hospital in Barcelona, Spain. All patients had received a histologic diagnosis of MC or FSGS lesion pattern in the years 2016 to 2024. The study was approved by the Ethical Committee on human experimentation of the Meyer Children's Hospital IRCCS, Florence, Italy, and Careggi University Hospital (AOUC; approval numbers 224/2021; 271/2022; and 7034/2011).

High-resolution confocal microscopy

High-resolution microscopy was performed as detailed in the Supplementary Methods.

STED microscopy

Stimulated emission depletion (STED) microscopy was performed as detailed in the Supplementary Methods.

ELISA for anti-nephrin autoantibodies

Enzyme-linked immunosorbent assay (ELISA) was performed as detailed in the Supplementary Methods.

Statistical analysis

Statistical analysis was performed as detailed in the Supplementary Methods.

RESULTS

IgG positivity of slit diaphragm occurs in a subset of patients with MC and FSGS lesions

To evaluate the possible binding of autoantibodies to the filtration slit, we labeled kidney sections for IgG and nephrin and imaged them by high-resolution confocal microscopy. We analysed 5 healthy kidneys and 128 kidney biopsies collected in the past 13 years at the Meyer Children's Hospital IRRCS in Florence, Italy and with a frozen sample available (Figure 1). All healthy controls were negative for IgG and positive for nephrin (Figure 1a). IgG deposits were present in patients with various forms of immune complex glomerulonephritis, such as IgA nephropathy, membranous nephropathy, lupus nephritis, and C3 glomerulopathy, as expected, but never localized along the slit stained by nephrin (Figure 1b–e). Among others, acute tubular necrosis was negative for IgG (Figure 1f). By contrast, high-resolution confocal microscopy identified IgG positivity on the slit diaphragm in some cases of MC or FSGS lesion patterns (Figure 1g–j). Overall, 30% of MC and 25% of FSGS showed IgG immunolabeling along the slit (Figure 1k). IgG deposits showed a granular or curvilinear pattern, as already previously reported,[7] sometimes even coexisting in the same biopsy (Figure 1i). As a further

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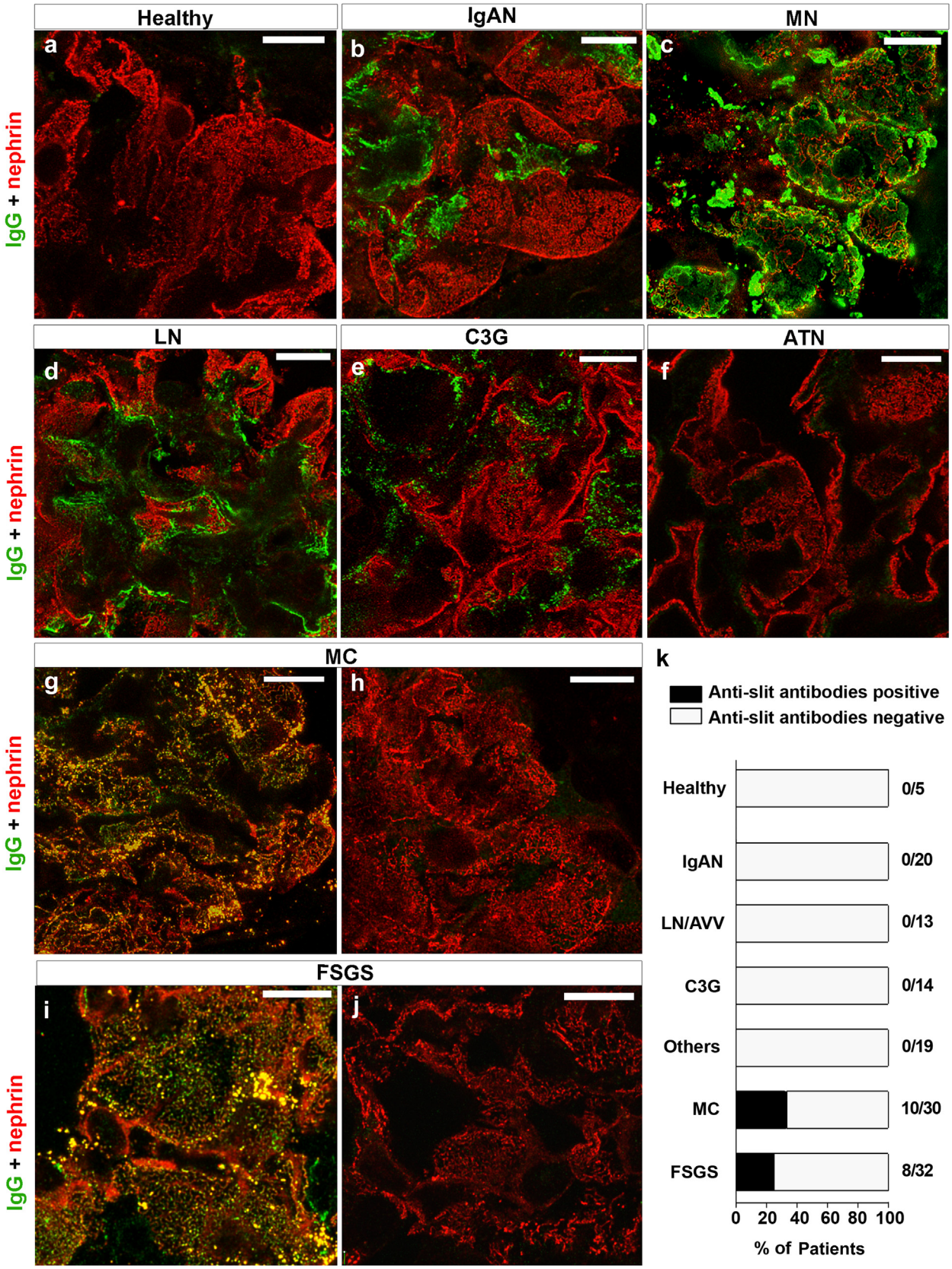

Figure 1 | IgG and nephrin immunolabeling in kidney biopsies with different lesion patterns. (**a–f**) Representative images of immunofluorescence staining for IgG (green) and nephrin (red) showing (**a,f**) the absence of IgG deposits on the slit diaphragm and/or (**b–e**) the complete lack of colocalization of IgG and nephrin in patients with different types of immune-complex (continued)

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confirmation, we also analyzed a cohort of adult patients with MC or FSGS lesion pattern at kidney biopsy (Supplementary Table S2). We observed IgG deposition on the slit diaphragm in 68.4% of MC and 19% of FSGS at kidney biopsy (Supplementary Figure S2A–E). Taken altogether, these results show that IgG localization along the filtration slit defines a subset of patients with podocytopathies and MC or FSGS lesion pattern, undistinguishable with routine pathology procedures.

IgG detected on the slit in a subset of MC or FSGS binds either nephrin or other slit antigens

IgG immunolabeling at the slit fully matched the location of nephrin in 14 of 18 cases (78%). Conversely, in 4 of the 18 cases (22.2%), there appeared to be a lack of IgG and nephrin colocalization at the slit. To definitively determine the presence or absence of IgG and nephrin colabeling across different instances, we used STED microscopy, achieving resolutions as fine as 60 nm. This method confirmed the observations of virtually complete overlap between IgG and nephrin at the filtration slit, as previously indicated by high-resolution microscopy (Figure 2a–d). Nonetheless, STED microscopy further confirmed cases where the colocalization of IgG with nephrin was negligible or nonexistent not only in pediatric (Figure 2e–h), but also in 44.4% of adult patients (Supplementary Figure S2F–G). Overall, this may suggest the presence of different types of anti-slit antibodies.

To further determine the specificity of the IgG detected on the slit diaphragm, we assessed the presence of anti-nephrin IgG in the serum by using 2 different immunoassays. To do this, we applied a commercially available ELISA assay for anti-nephrin antibodies to serum samples from the following groups of patients: (i) pediatric patients with nonproteinuric kidney diseases (n = 76); (ii) pediatric patients with steroid-sensitive nephrotic syndrome (SSNS) and active proteinuria who had not undergone kidney biopsy analysis (n = 13); and (iii) pediatric patients who had proteinuria at the moment of serum collection and showed deposition of anti-slit antibodies or had anti-slit antibodies negative at kidney biopsy (n = 12). The assay showed positivity in 4 of 6 serum samples of pediatric patients positive for anti-slit antibodies at kidney biopsy. Interestingly, the 2 patients negative in the anti-nephrin IgG ELISA serum assay but positive for anti-slit antibodies at kidney biopsy showed no nephrin colocalization of IgG deposited on the tissue. Moreover, all biopsy-proven anti-slit negative pediatric patients were negative in the serum (Figure 2i). The assay showed positivity of 5 of 13 pediatric patients affected by SSNS who had not undergone kidney biopsy, whereas the 76 controls were all negative (Figure 2i). To confirm these results, we reevaluated the serum samples from the 12 pediatric patients who had been proven to have anti-slit antibodies positive or negative at kidney biopsy using a second ELISA assay recognizing anti-nephrin antibodies directed against amino acids 23 to 1029 of nephrin, as previously reported. Importantly, the second ELISA assay for antibodies gave results virtually identical to those of the commercially available assay (Figure 2j). Assessment of serum samples in the second cohort of adult patients with MC and FSGS (n = 19) and age-matched controls (n = 17) analyzed gave similar results (Supplementary Figure S2H). A summary of all the pediatric and adult patients analyzed by ELISA is provided in Supplementary Figure S2I. Thus, STED microscopy and anti-nephrin IgG ELISA suggest that a subset of pediatric patients with MC and FSGS at biopsy have autoantibodies against either nephrin or nonnephrin slit antigens (i.e., anti-slit antibodies).

Patients with anti-slit antibodies show severe NS

We then analyzed the clinical phenotype of patients with MC or FSGS lesions with positive and negative staining for anti-slit antibodies (Supplementary Table S1). Pediatric patients with anti-slit antibodies showed a severe NS in most cases (Figure 3a). Hypoalbuminemia was <2 g/dl in 9 of 18 patients (50%), and hypercholesterolemia occurred in 17 of 18 patients (94.4%), of patients with anti-slit antibodies (Figure 3b and c). Finally, pediatric patients with anti-slit antibodies were frequently young, with 13 of 18 (72.2%) of cases having onset of NS before the age of 5 years (Figure 3d).

Presence of anti-slit antibodies associated with MC lesion pattern in 10 of 18 (55.6%) and with FSGS lesion pattern in the other 8 of 18 cases (44.4%; Figure 3e and Supplementary Figure S3A and B). Pediatric patients with anti-slit antibodies showed a lower rate of glomerulosclerosis (Figure 3f). This was still observed even when only patients with an FSGS lesion pattern at kidney biopsy were analyzed (Figure 3f). Consistently, they also showed less interstitial fibrosis and tubular atrophy (Supplementary Figure S3C). Electron microscopy showed that all pediatric patients with SRNS with anti-slit antibodies displayed complete fusion process effacement, consistent with the presence in the serum of a permeability factor, whereas anti-slit negative patients showed a variable degree of foot process effacement (Supplementary Figure S3D). Moreover, Kaplan-Meier analysis demonstrated that when the anti-slit antibodies status is known, the histologic pattern is not the main determinant of the outcome in terms of kidney failure (Figure 3g). Finally, pediatric patients with anti-slit antibodies were primary steroid resistant in 10 of 18 (55.6%) and secondary steroid resistant in 7 of 18

Figure 1 | (continued) glomerulonephritis. Bars = 10 μm. (**g–j**) Representative images of immunofluorescence staining showing the (**g,i**) presence or (**h,j**) absence of colocalization (yellow) of IgG (green) and nephrin (red) in some kidney biopsies with a histologic pattern of (**g,h**) minimal change (MC) or (**i,j**) focal segmental glomerular sclerosis (FSGS). Two collapsing glomerulopathies were included in FSGS. Bars = 10 μm. (**k**) Percentage of patients showing IgG immunolabeling along the slit. ATN, acute tubular necrosis; AVV, xxx; C3G, C3 glomerulopathy; IgAN, IgA nephropathy; LN, lupus nephritis; MN, membranous nephropathy. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

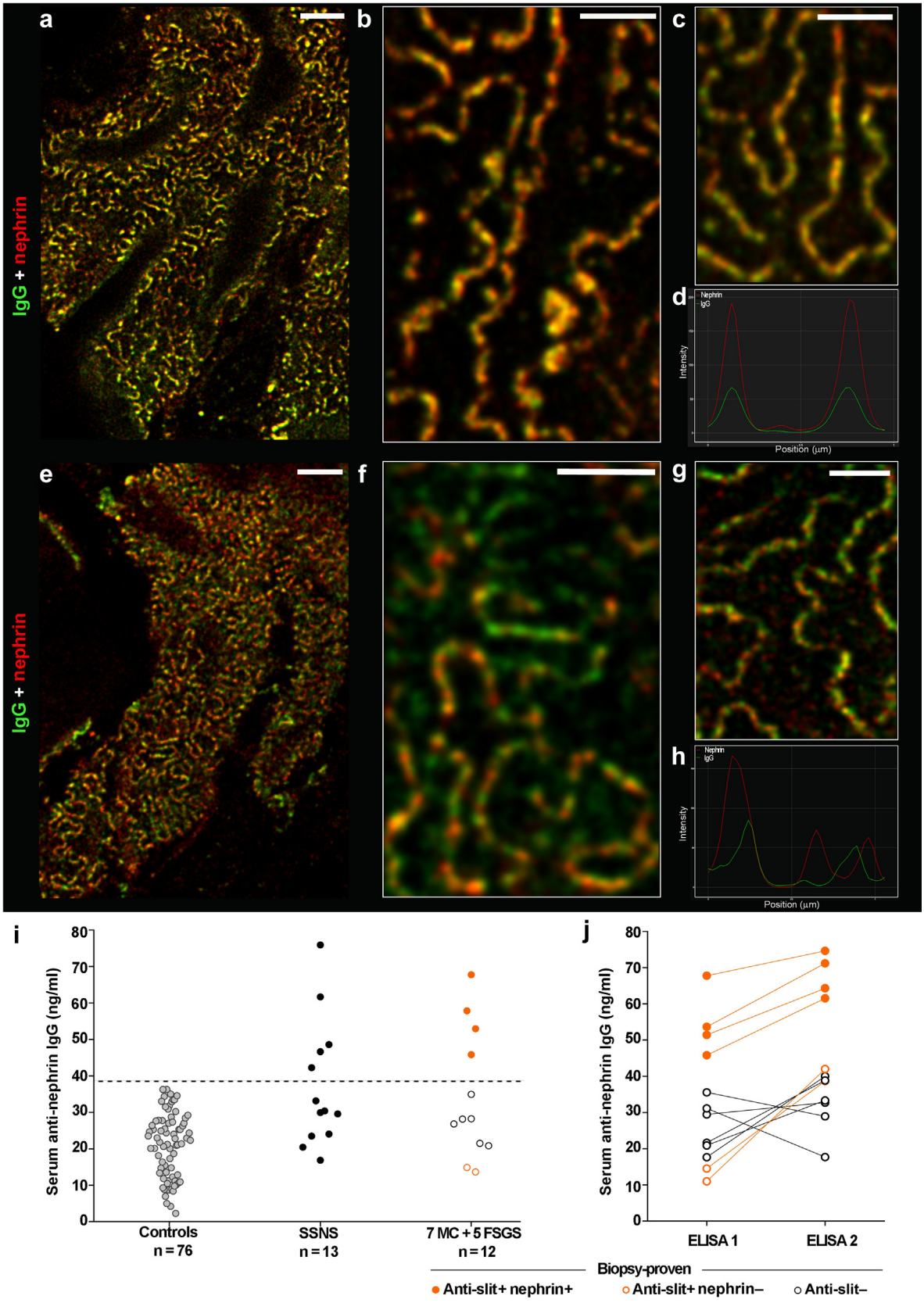

Figure 2 | Stimulated emission depletion (STED) microscopy and enxyme-linked immunosorbent assay (ELISA) analysis in representative patients. (**a**) Representative gated STED microscopy image of immunofluorescence staining showing colocalization (yellow) of IgG (green) and nephrin (red) in kidney biopsy. Bar = 2 µm. The image was deconvolved using using Huygens Professional software (24.04 version). (**b,c**) Higher magnifications showing a complete colocalization (yellow) between IgG (green) and nephrin (red). Bars = 1 µm. (**d**) (continued)

(38.9%) of cases (Figure 3g). Only 1 patient showed a subnephrotic proteinuria (Figure 3h). Clinical phenotype and histologic evaluation are presented in Supplementary Table S2. Taken altogether, these results show that patients with anti-slit antibodies usually present with a severe NS and only minor signs of chronic injury at biopsy.

Patients with anti-slit antibodies show the best prognosis among patients with SRNS

Pediatric patients with podocytopathies showing resistance after 4 weeks of steroid treatment have a variable renal prognosis, dependent on their response to second-line immunosuppressants.[1,2] Genetic patients are usually multidrug resistant and have a poor renal prognosis. Currently, we have no means to determine which patients will respond to second-line immunosuppressants until the results of genetic testing arrive, a process that can require several weeks/months for turnaround.[1,2]

Among the 45 patients with SRNS analyzed, genetic test had found a genetic cause in 12 of 45 (26.7%) patients (Supplementary Tables S3–S5). We identified anti-slit antibodies in 16 of 33 (48.5%; Figure 4a and c–d′) of nongenetic and in only 1 of 12 (8.3%; Figure 4b and e–f′) of genetic patients. The IgG-nephrin labeling status did not associate with either a MC or a FSGS lesion pattern (Figure 4a and b). Even among patients with SRNS, those with anti-slit antibodies showed a more severe NS at onset (Figure 4g and Supplementary Table S6). Interestingly 23 of 27 (85.2%) of patients with a protein/creatinine ratio >5 in our cohort were positive to anti-slit antibodies or had a genetic podocytopathy (Figure 4g). Although the response to renin-angiotensin system inhibitors was not different in anti-slit antibody positive versus negative patients (Supplementary Table S6), 12 of 13 (92.3%) antibody positive patients achieved remission with second-line immunosuppressant therapy versus only 2 of 10 (20%) antibody negative patients ($P = 0.001$; Figure 4h and Supplementary Table S6). Interestingly, the only anti-slit positive patient who was multidrug resistant (case 5) was a diagnosed case of Nail-Patella syndrome who developed proteinuria recurrence after kidney transplantation.[10,12,13] Kidney biopsy performed during 1 of these relapses confirmed the presence of anti-slit antibodies (Supplementary Figure S3E–G′). Among the other anti-slit antibody positive patients, only 1 of 16 (an obese patient who had undergone an episode of severe septic acute kidney injury) developed CKD, and no one reached kidney failure (Figure 4i). Kaplan-Meier analysis confirmed that nongenetic anti-slit positive patients had a favourable outcome; meanwhile, genetic patients, independently of anti-slit status, and nongenetic anti-slit negative patients showed similar poor prognosis at 10 years, both for achieving CKD and kidney failure (Figure 4i–j). Taken altogether, these results show that the presence of anti-slit antibodies at kidney biopsy identifies a subset of patients with an autoimmune podocytopathy who have a good response to second-line immunosuppressants and hence a better kidney outcome.

DISCUSSION

We had hypothesized that high-resolution microscopy could clearly identify patients with an autoimmune cause of their podocytopathy. We found the following: (i) Deposition of IgG antibodies on the slit diaphragm detectable with high-resolution microscopy identifies a subset of patients with MC or FSGS patterns at kidney biopsy. (ii) High-resolution microscopy on kidney biopsy as well as 2 ELISA assays suggested the presence of autoantibodies directed not only against nephrin, but also against other slit antigens (i.e., anti-slit antibodies). (iii) Patients with anti-slit antibodies usually show severe NS at onset but low signs of chronicity at kidney biopsy. (iv) Even among patients with SRNS, those anti-slit antibodies positive represent a definite subset that responds to second-line immunosuppressant therapy and have a better prognosis unless a concomitant genetic podocytopathy or other causes of CKD are present.

Watts *et al.*[7] were the first to report the presence of circulating anti-nephrin antibodies in some patients with NS and MC lesions, implying an autoimmune podocytopathy subset. A recent study confirmed this finding and extended it to detection of anti-nephrin antibodies in 9% of patients with primary FSGS.[14] We focused on their detection in frozen kidney tissue, which required either confocal microscopy with digital deconvolution techniques or STED microscopy. This technical approach is essential to resolve the nanometric size structures of slit diaphragm[15,16] and furthermore the antigen-antibody complexes that are neither part of the routine kidney pathology assessment nor yet available for paraffin-embedded tissue. Importantly, in this study, we provide detailed protocols for their assessment using these techniques (see Supplementary Methods).

Figure 2 | (continued) Representative fluorescence intensity profile plot showing the complete overlap between nephrin (red) and IgG (green) signals. (**e**) Representative gated STED microscopy image showing a minimal colocalization between IgG (green) and nephrin (red). Bar = 2 μm. (**f,g**) Higher magnifications showing a minimal or absent colocalization between IgG (green) and nephrin (red). Bars = 1 μm. (**h**) Representative fluorescence intensity profile plot showing the lack of overlap between nephrin (red) and IgG (green) signals. (**i**) Levels of anti-nephrin IgG in serum samples by using an ELISA assay for antibodies directed against Gln23-Gly92 amino acids of nephrin, in patients with biopsy-proven anti-slit positive nephrin positive (orange dots), biopsy-proven anti-slit positive nephrin negative (orange circles), patients affected by steroid-sensitive nephrotic syndrome (SSNS) who had not undergone to kidney biopsy (black dots), and controls (gray circles). The controls are both patients biopsy proven anti-slit negative and patients with nonproteinuric kidney disease. The dashed line represents the cutoff threshold for positivity determined as mean + 2 SD (22.72 + 17.17 = 39.88 ng/ml) of biopsy-proven anti-slit negative samples. (**j**) Comparison of levels of anti-nephrin IgG in serum samples assessed by using an ELISA assay for antibodies directed against Gln23∼Gly92 amino acids (ELISA 1) with levels assessed by using an ELISA assay for Gln23-Thr1029 amino acids (ELISA 2) of nephrin, in patients who underwent kidney biopsy. FSGS, focal segmental glomerular sclerosis; MC, minimal change. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

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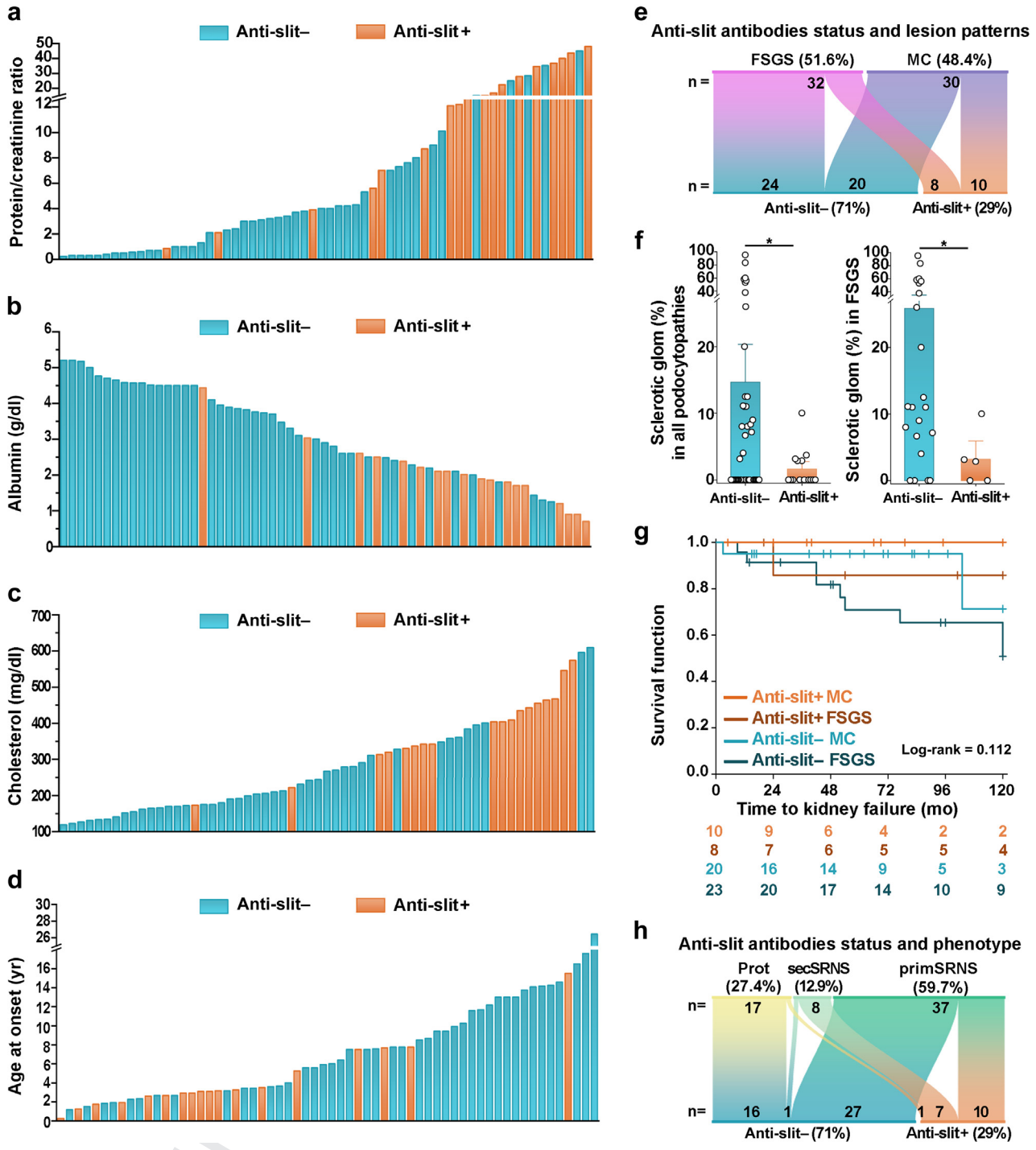

Figure 3 | Clinical phenotype and pathologic pattern, according to anti-slit status. (**a**) Distribution in ascending order of urinary protein/creatinine ratio (mg/mg) in patients with (orange) or without (light blue) anti-slit antibodies. (**b**) Distribution in decreasing order of serum albumin levels (g/dl) in patients with or without anti-slit antibodies. (**c**) Distribution in ascending order of serum cholesterol levels (mg/dl) in patients with or without anti-slit antibodies. (**d**) Distribution in ascending order of age at onset of patients with or without anti-slit antibodies. (**e**) Alluvial chart of patients with focal segmental glomerular sclerosis (FSGS) or minimal change (MC) lesion patterns (n = 62), showing the pathology pattern at kidney biopsy (above) and the anti-slit antibodies status as identified with high-resolution confocal microscopy (below). (**f**) Percentage of sclerotic glomeruli per section in all patients with podocytopathies (on the left, $P = 0.04$) and in only patients with FSGS lesion pattern (on the right, $P = 0.03$). (**g**) Kaplan-Meier curves of the time from the onset to kidney failure over a period of 10 years of follow-up, compared by log-rank test. Patients are stratified according to anti-slit status and histologic lesion. (+) Censored observations. One patient with kidney failure at the onset was not included in the analysis. (**h**) Alluvial chart of patients with subnephrotic proteinuria (Prot) or primary or secondary steroid-resistant nephrotic syndrome (primSRNS or secSRNS, respectively; n = 62), showing the clinic phenotype (above) and the anti-slit antibodies status as identified with high-resolution confocal microscopy (below).

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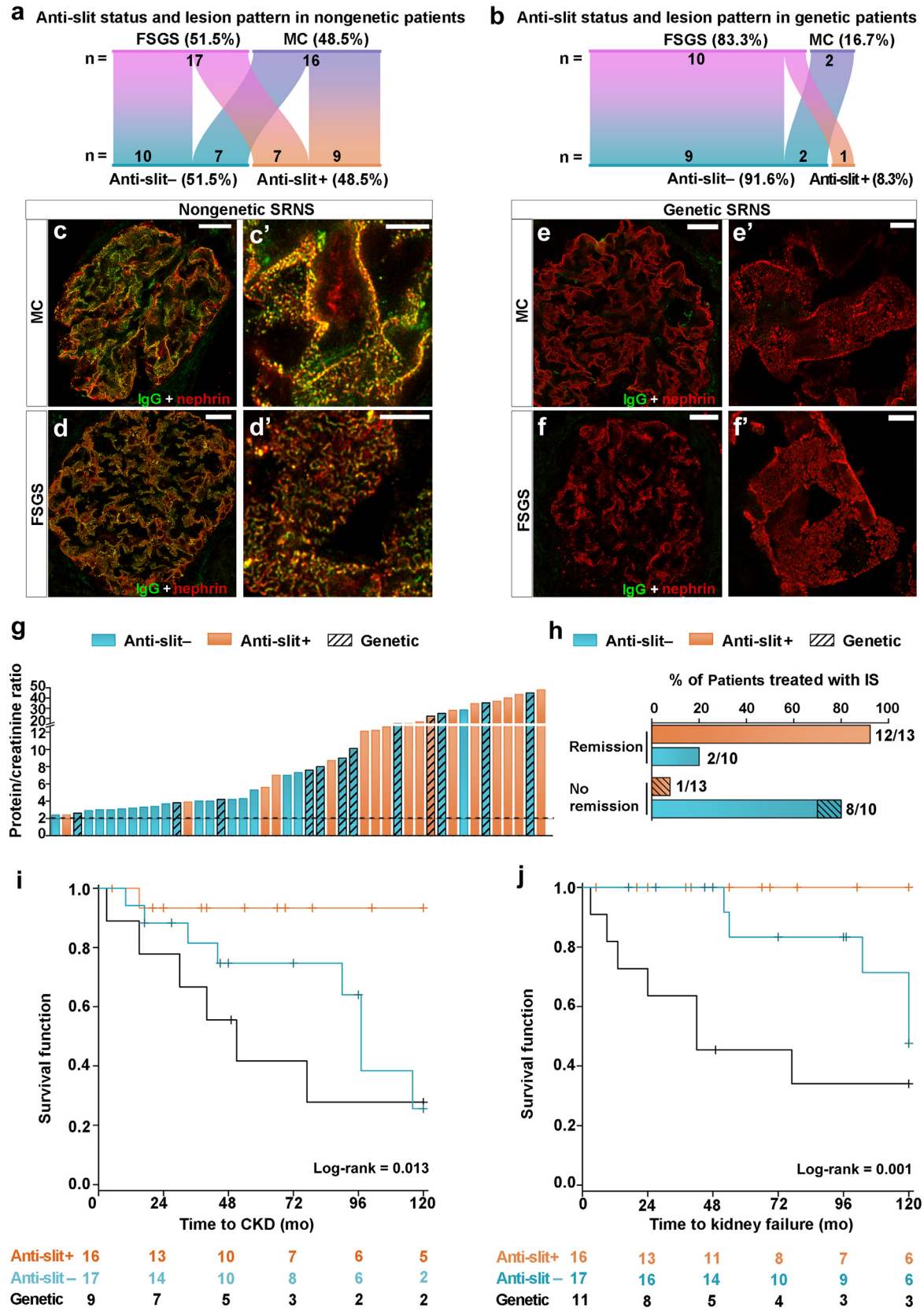

Figure 4 | Anti-slit antibodies status, response to treatment, and outcome in steroid-resistant nephrotic syndrome (SRNS). (a,b) Alluvial charts of patients with (**a**) nongenetic SRNS (n = 33) and (**b**) genetic SRNS (n = 12), showing the pathology pattern at kidney biopsy (above) and the anti-slit antibodies status as identified with high-resolution confocal microscopy (below). (**c,d**) Representative (continued)

Our results independently confirm nephrin autoimmunity as a cause of NS. However, STED microscopy also showed IgG deposits on the slit diaphragm not colocalized with nephrin, suggesting the presence in approximately one-fourth of pediatric patients with SRNS of autoimmunity directed toward other antigen(s). Two ELISA assays were consistent in detecting anti-nephrin antibodies in the serum of patients who had IgG deposits colocalized with nephrin, and in not finding them in those where IgG deposits did not colocalize with nephrin on kidney biopsy. The nature of the other antigen(s) in addition to nephrin remains to be established. However, detection of IgG on kidney biopsy by high-resolution microscopy permits us to identify the patients with autoimmune forms of NS, independently from the antigen of the slit involved. Some patients who were negative for anti-slit antibodies still responded to immunosuppressive treatment, suggesting other immune mechanisms may also be involved. Anyway, the results of this study have the important clinical implication that appropriate decisions about treatment cannot be taken based on serum anti-nephrin IgG ELISA only, as this approach would miss cases with autoimmune podocytopathies directed against other slit antigens. Moreover, we report the presence of anti-slit antibodies in a higher percentage of patients with FSGS than previously reported (25% of pediatric and 19% of adult considering total cases, both primary and secondary). These results suggest that other antigens of the slit are involved as causes of autoimmune podocytopathies, beyond those caused by anti-nephrin antibodies. In these cases, STED microscopy could help in their identification. Indeed, screening of patients with anti-slit positivity on the biopsy and anti-nephrin negativity in the circulation for colabeling with a panel of slit proteins could help to identify further slit autoantigens.

In our study, the cohort was biased for SRNS, because these are the only pediatric patients who have indication to kidney biopsy,[1,2,17] which may also explain a lower rate of positivity for anti-nephrin antibodies, as reported in other cohorts.[14] Indeed, pediatric patients with SSNS can be in most cases successfully treated with steroids with favorable outcome, so they usually do not require a sophisticated diagnostic workup or even a kidney biopsy.[1,2,18] By contrast, pediatric patients who do not respond to steroids, primarily or secondarily, represent a heterogeneous group involving genetic and nongenetic cases that require fast and appropriate diagnosis to get the right treatment.[2,16,19] Interestingly, in our cohort, 87.5% of secondary steroid-resistant pediatric patients showed positivity for anti-slit antibodies, which is consistent with the high rate of proteinuria recurrence after kidney transplantation in this subgroup when they reach kidney failure.[6,20,21] Thus, the differential diagnosis between autoimmune, genetic, infectious, or toxic podocytopathy matters especially in these patients. In particular, the podocytopathy lesion patterns of MC or FSGS do not inform about treatment choices, because they are unspecific as once more demonstrated by us in terms of an autoimmune cause.[1,10] By contrast, even in SRNS, anti-slit autoimmunity connects with the response to (second-line) immunosuppressant therapies, similar to patients with SSNS.[22] We show that anti-slit autoimmunity occurs in patients with SSNS and SRNS, and more rarely can even occur in patients with subnephrotic proteinuria. This requires a gradient of responses to different levels of immunosuppression related to the size and affinity of nephrin-reactive T, B, and plasma cell clones, as reported for other autoimmune diseases, including autoimmune forms of glomerulonephritis.[23,24]

Interestingly, in kidney biopsy, the presence of anti-slit antibodies correlated rather well with a lower chronicity index, detected as amount of glomerulosclerosis, interstitial fibrosis, and tubular atrophy, and was a good predictor of kidney outcome irrespective of the lesion pattern (MC or FSGS) at kidney biopsy. This observation directly mirrors the positive response to therapy and better clinical outcome observed in this group of patients.[1,18] However, we speculate that this may also associate with the pathogenic mechanisms of the disease. Indeed, these autoantibodies determine NS binding to slit proteins that are essential to maintain the integrity of the glomerular filtration barrier. In these patients, disruption of this integrity, reflected by foot process effacement at electron microscopy, is usually not associated with podocyte loss, and thus always potentially reversible without permanent glomerular damage, even in presence of severe NS. By contrast, in patients with genetic podocyte abnormalities, disruption of the glomerular filtration barrier with foot process effacement is irreversible, and severe NS occurs in association with podocyte loss, determining glomerulosclerosis and secondary interstitial fibrosis, explaining the negative prognosis.[1,23] Consistently, the only patient with anti-slit antibodies of this cohort reaching kidney failure also

Figure 4 | (continued) images of immunofluorescence staining showing colocalization (yellow) of IgG (green) and nephrin (red) in kidney biopsy from patients with nongenetic SRNS and a histologic pattern of (**c**) minimal change (MC) or (**d**) focal segmental glomerular sclerosis (FSGS). Bars = 20 μm. (**c′,d′**) Higher-magnification details are shown. Bars = 5 μm. (**e,f**) Representative images of immunofluorescence staining showing the lack of IgG (green) along the slit (nephrin stained in red) in kidney biopsy from patients with genetic SRNS and a histologic pattern of (**e**) MC or (**f**) FSGS. Bars = 20 μm. (**e′,f′**) Higher-magnification details are shown. Bars = 5 μm. (**g**) Distribution in ascending order of urinary protein/creatinine ratio (mg/mg) in patients with (orange) or without (light blue) anti-slit antibodies and in patients with genetic SRNS (black lines). (**h**) Percentage of patients with or without anti-slit antibodies and patients with genetic SRNS who achieved remission or not with second-line immunosuppressant therapy. (**i,j**) Kaplan-Meier curves of the time (**i**) from the onset to chronic kidney disease (CKD) grade 2 (G2) and (**j**) from the onset to kidney failure over a period of 10 years of follow-up, compared by log-rank test. Patients with SRNS are stratified according to anti-slit status and according to the genetic status. (+) Censored observations. Patients with CKD ≥ G2 (n = 3) and kidney failure (n = 1) at the onset were not included in the analysis. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

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showed a concomitant genetic disease. This observation suggests the need of an intensified immunosuppression in patients with steroid-resistant autoimmune podocytopathies, and particularly of anti–B-cell treatment, not only in general but also when concomitant risk factors for CKD progression are present. This is in agreement with recent observations reporting anti-nephrin antibodies in relapsing pediatric patients after kidney transplantation.[7,8]

This study has some limitations. First, the availability of high-resolution microscopy is limited and not part of clinical practice, potentially hindering the widespread use of this test. However, the detailed protocols provided in this article for adapting confocal microscopes to detect anti-slit antibodies may facilitate broader adoption. Second, the number of patients who could be validated as positive for anti-slit antibodies but negative for anti-nephrin antibodies with STED microscopy as well as the 2 ELISA assays was small (only 6). Despite this, STED microscopy was applied to all available kidney biopsies, indicating that 22% of pediatric and 44% of adult patients with autoimmune podocytopathies may be positive for anti-slit antibodies other than anti-nephrin. Third, we were unable to compare our ELISA assays with the one used by Hengel *et al.*,[14] which was recently used for detecting anti-nephrin antibodies in >500 patients but is not commercially available. Nevertheless, our consistent results across different ELISA assays and high-resolution confocal and STED microscopy validate our observations. In addition, our data obtained with ELISA assays report a positivity for anti-nephrin antibodies of 57% among pediatric patients with active MC, but of only 12.5% of adult patients with primary FSGS. This is consistent with findings of Hengel *et al.*,[14] that in their pediatric cohorts found positivity for anti-nephrin antibodies in 52% and 69% of children with idiopathic nephrotic syndrome or active MC, respectively, but in only 9% of adult patients with primary FSGS, corroborating the existence of other antigens in patients with autoimmune podocytopathies, particularly in primary FSGS. Further studies with larger patient cohorts are needed to confirm these findings and identify other potential antigens involved.

These results have a series of important clinical implications: (i) High-resolution microscopy on kidney biopsies is a novel analytical tool to identify autoimmune podocytopathies, independently from the lesion pattern at biopsy. (ii) Anti-slit autoimmunity identifies a subset of patients with SRNS who will respond to second-line immunosuppressants with a better kidney prognosis, because autoimmune podocytopathies can be controlled like other autoantibody-mediated autoimmune disease, such as pemphigus, idiopathic thrombocythemia, or anti–phospholipase A2 receptor–glomerulonephritis. (iii) Anti-slit autoimmunity not responding to immunosuppressants may relate to a concomitant genetic nephropathy or other cofactors for CKD progression that determine kidney outcome.

DISCLOSURE

XXX.

DATA STATEMENT

The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Material.

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Supplementary material is available online at www.kidney-international.org.

REFERENCES

1. Kopp JB, Anders HJ, Susztak K, et al. Podocytopathies. *Nat Rev Dis Primers*. 2020;6:68.
2. Kidney Disease: Improving Global Outcomes (KDIGO) Glomerular Diseases Work Group. KDIGO clinical practice guidelines for management of glomerular disease. *Kidney Int*. 2021;100(4S):S1–S276.
3. Landini S, Mazzinghi B, Becherucci F, et al. Reverse phenotyping after whole-exome sequencing in steroid-resistant nephrotic syndrome. *Clin J Am Soc Nephrol*. 2020;15:89–100.
4. Trautmann A, Schnaidt S, Lipska-Ziętkiewicz BS, et al. Long-term outcome of steroid-resistant nephrotic syndrome in children. *J Am Soc Nephrol*. 2017;28:3055–3065.
5. Mann N, Braun DA, Amann K, et al. Whole-exome sequencing enables a precision medicine approach for kidney transplant recipients. *J Am Soc Nephrol*. 2019;30:201–215.
6. Mason AE, Sen ES, Bierzynska A, et al. Response to first course of intensified immunosuppression in genetically stratified steroid resistant nephrotic syndrome. *Clin J Am Soc Nephrol*. 2020;15:983–994.
7. Watts AJB, Keller KH, Lerner G, et al. Discovery of autoantibodies targeting nephrin in minimal change disease supports a novel autoimmune etiology. *J Am Soc Nephrol*. 2022;33:238–252.
8. Shirai Y, Miura K, Ishizuka K, et al. A multi-institutional study found a possible role of anti-nephrin antibodies in post-transplant focal segmental glomerulosclerosis recurrence. *Kidney Int*. 2024;105:608–617.
9. Becherucci F, Landini S, Palazzo V, et al. A clinical workflow for cost-saving high-rate diagnosis of genetic kidney diseases. *J Am Soc Nephrol*. 2023;34:706–720.
10. Giglio S, Provenzano A, Mazzinghi B, et al. Heterogeneous genetic alterations in sporadic nephrotic syndrome associate with resistance to immunosuppression. *J Am Soc Nephrol*. 2015;26:230–236.
11. Kidney Disease: Improving Global Outcomes (KDIGO) CKD Work Group. KDIGO 2024 Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease. *Kidney Int*. 2024;105(4S):S117–S314.
12. Lazzeri E, Ronconi E, Angelotti ML, et al. Human urine-derived renal progenitors for personalized modeling of genetic kidney disorders. *J Am Soc Nephrol*. 2015;26:1961–1974.
13. Bongers EM, Huysmans FT, Levtchenko E, et al. Genotype-phenotype studies in Nail-Patella syndrome show that LMX1B mutation location is involved in the risk of developing nephropathy. *Eur J Hum Genet*. 2005;13:935–946.
14. Hengel FE, Dehde S, Lassé M, et al., International Society of Glomerular Disease. Autoantibodies targeting nephrin in podocytopathies. *N Engl J Med*. 2024;391:422–433.
15. Unnersjö-Jess D, Butt L, Höhne M, et al. A fast and simple clearing and swelling protocol for 3D in-situ imaging of the kidney across scales. *Kidney Int*. 2021;99:1010–1020.
16. Angelotti ML, Antonelli G, Conte C, et al. Imaging the kidney: from light to super-resolution microscopy. *Nephrol Dial Transplant*. 2021;36:19–28.
17. Trautmann A, Vivarelli M, Samuel S, et al., International Pediatric Nephrology Association. IPNA clinical practice recommendations for the

diagnosis and management of children with steroid-resistant nephrotic syndrome. *Pediatr Nephrol*. 2020;35:1529–1561.
18. Kyrieleis HA, Löwik MM, Pronk I, et al. Long-term outcome of biopsy-proven, frequently relapsing minimal-change nephrotic syndrome in children. *Clin J Am Soc Nephrol*. 2009;4:1593–1600.
19. Tullus K, Webb H, Bagga A. Management of steroid-resistant nephrotic syndrome in children and adolescents. *Lancet Child Adolesc Health*. 2018;2:880–890.
20. Ding WY, Koziell A, McCarthy HJ, et al. Initial steroid sensitivity in children with steroid-resistant nephrotic syndrome predicts post-transplant recurrence. *J Am Soc Nephrol*. 2014;25:1342–1348.
21. Bierzynska A, McCarthy HJ, Soderquest K, et al. Genomic and clinical profiling of a national nephrotic syndrome cohort advocates a precision medicine approach to disease management. *Kidney Int*. 2017;91:937–947.
22. Larkins NG, Liu ID, Willis NS, et al. Non-corticosteroid immunosuppressive medications for steroid-sensitive nephrotic syndrome in children. *Cochrane Database Syst Rev*. 2020;4:CD002290.
23. Anders HJ, Kitching AR, Leung N, et al. Glomerulonephritis: immunopathogenesis and immunotherapy. *Nat Rev Immunol*. 2023;23:453–471.
24. Fervenza FC, Appel GB, Barbour SJ, et al. Rituximab or cyclosporine in the treatment of membranous nephropathy. *N Engl J Med*. 2019;381:36–46.