Rituximab is a safe and effective long-term treatment for children with steroid and calcineurin inhibitor–dependent idiopathic nephrotic syndrome

Pietro Ravani[1], Alessandro Ponticelli[2], Chiara Siciliano[2], Alessia Fornoni[3], Alberto Magnasco[2], Felice Sica[4], Monica Bodria[2], Gianluca Caridi[2], Changli Wei[3], Mirco Belingheri[5], Luciana Ghio[5], Sandra Merscher-Gomez[3], Alberto Edefonti[5], Andrea Pasini[6], Giovanni Montini[6], Corrado Murtas[2], Xiangyu Wang[1], Daniel Muruve[1], Augusto Vaglio[7], Davide Martorana[7], Antonello Pani[8], Francesco Scolari[9], Jochen Reiser[10] and Gian M. Ghiggeri[2]

[1]Division of Nephrology, Department of Medicine, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada; [2]Division of Nephrology, Dialysis, Transplantation, and Laboratory on Pathophysiology of Uremia, Istituto G. Gaslini, Genoa, Italy; [3]Division of Nephrology and Hypertension, Department of Medicine, University of Miami Miller School of Medicine, Miami, Illinois, USA; [4]Division of Pediatrics, Hospital of Foggia, Foggia, Italy; [5]Pediatric Nephrology and Dialysis Unit, Fondazione IRCCS Ca' Granda, Ospedale Maggiore, Policlinico, Milan, Italy; [6]Nephrology and Pediatric Dialysis, Department of Pediatrics, Azienda Ospedaliera, Universitaria Sant'Orsola, Bologna, Italy; [7]Dipartimento di Clinica Medica e Nefrologia, Azienda Ospedaliero-Universitaria di Parma, Parma, Italy; [8]Nefrologia e Dialisi, Azienda Ospedaliera G. Brotzu, Cagliari, Italy; [9]Division of Nephrology and Dialysis, Ospedale di Montichiari, Brescia, Italy and [10]Department of Medicine, Rush University, Chicago, Illinois, USA

In children with idiopathic nephrotic syndrome, rituximab can maintain short-term remission with withdrawal of prednisone and calcineurin inhibitors. Long-term effects including the number of repeated infusions to maintain remission are unknown. To test this, we treated 46 consecutive children with idiopathic nephrotic syndrome lasting for at least 1 year (mean 6.3 years), maintained in remission with oral prednisone and calcineurin inhibitors. They received 1–5 rituximab courses during a median follow-up of 3 years. Oral agents were tapered after each infusion, and completely withdrawn within 45 days. Rituximab was well tolerated. Six-month probabilities of remission were 48% after the first infusion and 37% after subsequent infusions. One- and 2-year-remission probabilities were, respectively, 20 and 10%. Median time intervals between complete oral-agent withdrawal and relapse were 5.6 and 8.5 months, respectively, following the first and subsequent courses. The time to reconstitution of CD20 cells correlated with the duration of remission, but was not associated with variation in FcyR, CD20, or *SMPDL-3B* polymorphisms. Podocyte Src phosphorylation was normal. Thus, rituximab can be safely and repeatedly used as a prednisone and calcineurin inhibitor–sparing therapy in a considerable proportion of children with dependent forms of idiopathic nephrotic syndrome. Further study is needed to identify patients who will benefit most from rituximab therapy.

Idiopathic nephrotic syndrome (INS) affects 2–3 new children per 100,000 children/year, and it is the most common kidney disease in the pediatric population after congenital abnormalities of the urinary tract and cystic disorders.[1] Although 90% of these children favorably respond to steroids, the relapse rate is as high as 80%, and a long-term combination of steroids and calcineurin inhibitors is often required to maintain remission.[2,3] Disease remission and prevention of kidney disease progression are the main long-term care objectives.[4] Given the toxicity of these agents, however, oral agent–free follow-up periods are attractive short-term goals.[2,5,6]

Rituximab (Mabthera)[R] is a monoclonal antibody directed against CD20, a 35-kDa protein highly expressed on B lymphocytes from early to late B-cell stages.[7,8] CD20 expression in most B-cell lymphomas supports its use for hematological malignancies.[9,10] Studies in these settings showed that rituximab is safe in the mid-term, with most adverse events being limited to fever and chills at the first infusion. Nephrologists started to use rituximab in children with INS following the observation of an anti-proteinuric effect in patients with focal and segmental glomerulosclerois associated with post-transplant lymphoproliferative disorders who were treated with rituximab.[11,12] Although rituximab

may not be effective in cortico-resistant forms of INS,[13] data from several small case series[14–16] and a randomized controlled trial[17] indicate that rituximab may successfully replace steroids and calcineurin inhibitors in children with INS whose remission state is dependent on both these drugs. In these forms of INS, rituximab may be as effective as the combination of steroids and calcineurin inhibitors in maintaining short-term remission, allowing withdrawal of the oral therapy for 9 months in 50% of cases.[16,17]

Despite these promising results, additional data are needed to inform clinical practice on how best to use rituximab in dependent forms of INS. For example, it is unknown whether individual characteristics and additional rituximab doses may favorably affect response duration and prolong disease remission.[18] INS tends to relapse and long-term data are necessary on factors affecting frequency and number of infusions, and patient tolerance or toxicity. Although the side effects and discomfort associated with administration can be minimized with pretreatment and by slowing the infusion speed, more severe complications such as fatal pulmonary fibrosis[19] and progressive multifocal leukoencephalopathy[20,21] remain a concern.

Here we present long-term follow-up data from children with INS dependent on both prednisone and calcineurin inhibitors for at least 1 year and in remission for at least 6 months. Consecutive children with these characteristics were treated with one or more rituximab infusions (up to 5) after the completion of a published clinical trial,[17] either because they had signs of toxicity from prednisone or calcineurin inhibitors or to prevent toxicity following treatment for at least 1 year. We conducted clinical and vitro studies to describe the following: (1) the probability of maintaining disease remission for 6 and 12 months after rituximab infusion and prednisone and calcineurin-inhibitor withdrawal; (2) the risk of disease relapse in children who remained in remission after rituximab infusion and prednisone and calcineurin-inhibitor withdrawal; (3) the toxicity profile of repeated rituximab infusions; and (4) the relationship between variation in FcyR, CD20, and/or SMPDL-3B polymorphisms and response to rituximab.

RESULTS
Patient characteristics
Baseline characteristics of the study cohort ($N = 46$) are summarized in Table 1. All children had INS dependent on oral prednisone and calcineurin inhibitors for at least 1 year (6.3 ± 4.1 years), and repeatedly failed to withdraw oral agents (relapsing within 1–2 weeks of withdrawal). These children were maintained in disease remission with average oral doses of prednisone and calcineurin inhibitors (Table 1). All had normal creatinine clearance, serum albumin, and cholesterol. Children were on average 10 years old and tended to be male (63%). Disease duration strongly correlated with age ($\rho = 0.82$; $P < 0.001$). Signs of toxicity were present in 17 children and were significantly more likely in older children (Supplementary Table S1 online). The use of angiotensin

Table 1 | Baseline characteristics by age above and below the sample median (9.4 years)

	All	Age $\leqslant 9.4$	Age > 9.4	
	$N = 46$	$N = 23$	$N = 23$	P-value
Ages (year)	9.9 ± 4.3	6.5 ± 2.0	13.4 ± 3.1	< 0.01
Creatinine clearance (ml/min)	98 ± 13	96 ± 10	100 ± 15	0.29
Total cholesterol (mg/dl)	181 ± 15	182 ± 16	180 ± 15	0.66
Serum albumin (g/dl)	3.6 ± 0.4	3.5 ± 0.4	3.6 ± 0.4	0.40
Duration years (mean ± s.d.)	6.3 ± 4.1	3.4 ± 2.0	9.2 ± 3.5	< 0.01
1–3 years (%)	11 (24)	11 (48)	0 (0)	< 0.01
3–6 years (%)	17 (37)	12 (52)	5 (22)	
>6 years (%)	18 (39)	0 (0)	18 (78)	
Body weight	37.1 ± 15.6	25.7 ± 9.3	48.4 ± 11.9	< 0.01
Renal histology (n°)	16	5	11	0.999
Not performed	30	18	12	
FSGS	2	0	2	
MCN	10	4	6	
IgM	4	1	3	
Prednisone (mg/kg/day)	0.4 ± 0.3	0.5 ± 0.3	0.3 ± 0.2	0.04
CyA (mg/kg/day; n)	2.5 ± 1.2 (17)	2.9 ± 1.2	2.3 ± 1.2	0.277
FK (mg/kg/day; n)	0.12 ± 0.02 (19)	0.11 ± 0.005	0.12 ± 0.01	
Male	29 (63%)	16 (69%)	13 (56%)	0.359
Toxicity	17 (37%)	4 (17%)	13 (56%)	< 0.01
ACEI/ARB	31 (67%)	17 (74%)	14 (61%)	0.345

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CyA, cyclosporine A; FK, tacrolimus; FSGS, focal segmental glomerulosclerosis; MCN, minimal change nephropathy.
Most data are given as mean ± s.d.

receptor blockers or angiotensin-converting enzyme inhibitors was similar across age groups.

Probability of remission
During an average follow-up of 3 years (range 1–5), the study participants received one- to five courses of rituximab: all received one; 39 received two; 15 received three; 3 received four; and 1 received five courses (Figure 1). Children were considered to be in remission for at least 6 months after rituximab infusion if they (1) successfully completed prednisone and calcineurin-inhibitor tapering, and (2) remained in stable remission (see Materials and Methods) without oral agents from the day of complete drug withdrawal. We also considered a 12-month remission, and single oral-agent withdrawal and withdrawal of both oral agents. Figure 2 shows the distribution of 6- and 12-month remissions after the initial treatment (top), and after any treatment (bottom), according to the oral agents that were withdrawn. At 6 months, 22 children (48%) were in remission without oral agents; 25 (54%) were in prednisone-free remission (three restarted calcineurin inhibitors); and 27 (59%) were in calcineurin inhibitor–free remission (five restarted prednisone). Probabilities of 6-month remission following subsequent treatments were not statistically
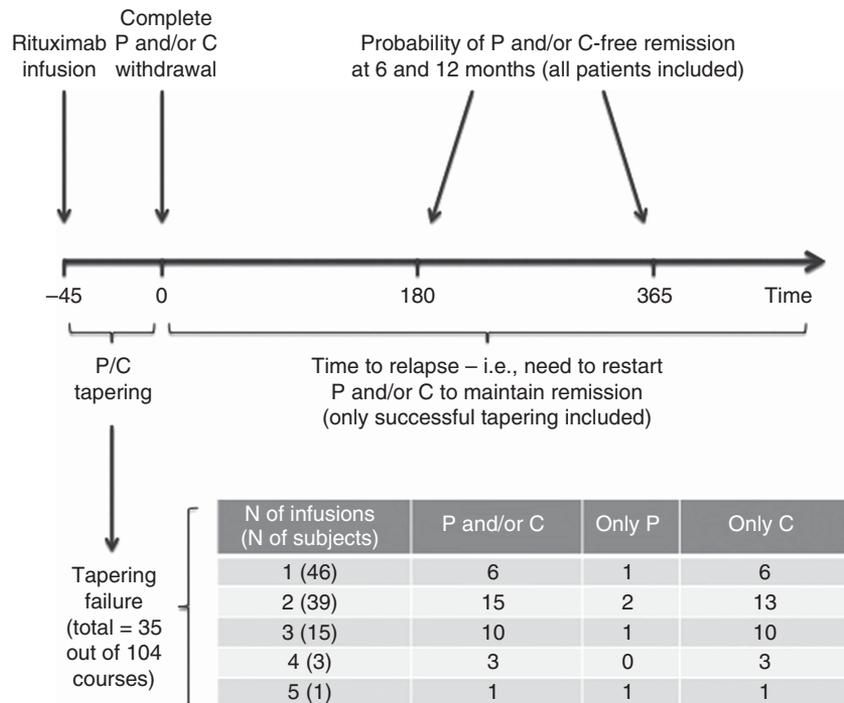
Figure 1 | Outcome definitions: we studied the probability of prednisone (P) and calcineurin (C) inhibitor–free remission (top) for at least 6 and 12 months (primary outcomes; no children were excluded from these analyses). Remission (absence of proteinuria) could be attained with complete withdrawal of both P and C inhibitors or either of them. We also studied time to relapse (proteinuria or need to reintroduce P or C inhibitors) after successful tapering (bottom). This analysis only included children who completed drug withdrawal, as children who failed to complete drug withdrawal could not be considered at risk for relapse (secondary outcome).

different. After 1 year from any treatment, ∼20% of children were still in prednisone and calcineurin inhibitor–free remission, and 25% of them were in single oral agent–free remission. All these children were still in remission at 18 months, and half of them remained in remission for 24 months. Probabilities of single and dual oral agent–free remission at 6 months were significantly higher ($P < 0.001$) in children older than 9.4 years than in those who were younger (68 vs. 32% for prednisone and calcineurin inhibitor–free remission; 62 vs. 38% for prednisone-free remission; and 65 vs. 35% for calcineurin inhibitor–free remission). In adjusted analyses (Table 2), age at diagnosis was the strongest predictor of drug-free remission for 6 and 12 months. Each year of age was associated with a 71% and 31% greater probability of prednisone and calcineurin inhibitor–free remission for 6 and 12 months, respectively. Results were similar in models of single oral agent–free remission for 6 and 12 months (Table 2). We found no difference between children who did and did not participate in the trial and between those who did and did not have signs of drug toxicity.

Risk of relapse

We studied the duration of the remission state as the time interval between complete oral-agent withdrawal after the first and subsequent rituximab infusions and relapse of nephrotic syndrome or the need to restart oral agents, or

study end (see Materials and Methods). Tapering failures ($N = 35$) were excluded from this analysis (Figure 1). Figure 3 summarizes relapse-free survival probabilities according to whether remission was free from both oral agents, free from prednisone only, and free from calcineurin inhibitors only. Median survival times were 5.6–7.3 months after the first treatment and 8.5–11.6 months after the second and subsequent treatments (Table 3). Each year of age at diagnosis predicted longer relapse-free survival (Supplementary Table S2 online).

Other follow-up data

CD20 count. CD20 remained undetectable for 195 days (s.d. 90) after the first treatment and for 219 days (s.d. 142) after the second and subsequent treatments ($P = 0.444$). Adjusting for age, CD20 reappearance was on average 185 days (95% CI from 104 to 267) in children who achieved prednisone and calcineurin inhibitor–free remission for 6 months versus those who did not (109 days; 45–172; $P = 0.01$), 152 days (76–227) in children who achieved prednisone-free remission for 6 months versus those who did not (96 days; 27–165; $P = 0.06$), and 161 days (77–246) in children who achieved calcineurin inhibitor–free remission for 6 months versus those who did not (109 days; 45–173; $P = 0.01$).

Adverse events. Five patients required rituximab infusion in intensive care unit for initial bronchospasm, which
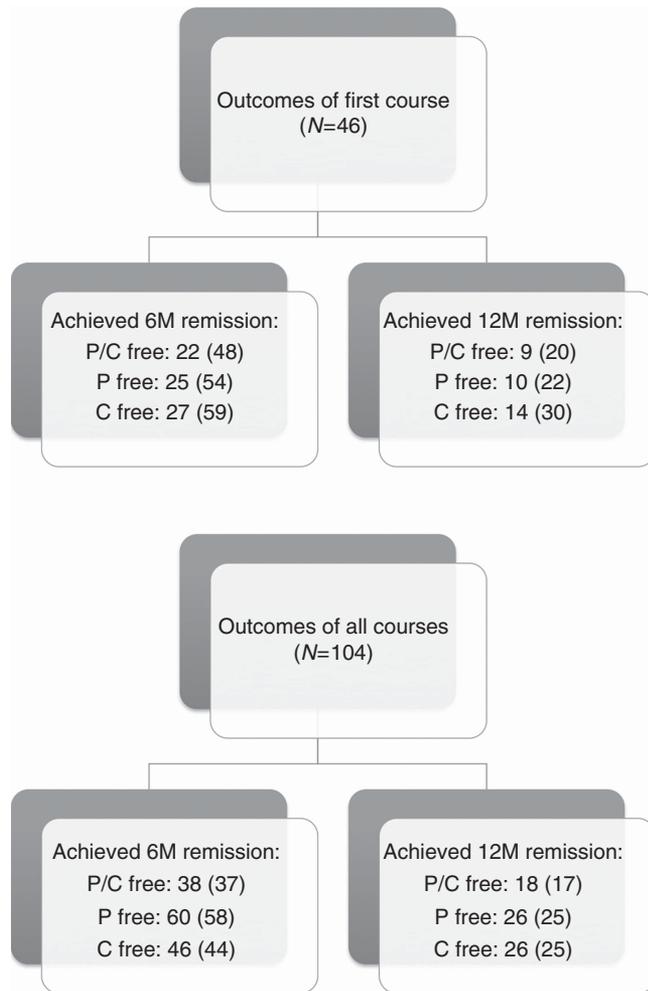
Figure 2 | Distribution of 6- and 12-month remissions after the initial treatment (top), and after any treatment (bottom), according to the drugs that were successfully withdrawn. Absolute frequencies (%) are reported for each outcome. 6M and 12M remissions, remission for 6 and 12 months; P/C, prednisone and calcineurin inhibitors–free remission; P, prednisone-free remission; C, calcineurin inhibitor–free remission.

improved after slowing the infusion rate. No further medication was necessary. Three patients presented fever with migrating skin rash and acute arthritis appearing 2–4 weeks from infusion that rapidly resolved with nonsteroidal anti-inflammatory medications. Two patients had neutropenia associated with transient viral infection. One patient (with known vesicoureteral reflux) developed acute pyelonephritis with neutropenia and negative markers for viral infection.

Cost data. See Supplementary Results online.

Laboratory data

***FCGR2B* and *FCGR3A* polymorphisms.** *FCGR2B* and *FCGR3A* polymorphisms affect rituximab binding to natural killer cells and drive its antibody-dependent cellular toxicity.[22] The comparison of FCGR2A rs1801274 SNP allelic and

Table 2 | Effect of age (per year) on the probability of remission for 6 and 12 months

	6 Months		12 Months	
	OR	95% CI	OR	95% CI
Both P and C	**1.71**	**1.11–2.62**	1.31	1.04–1.65
Prednisone	1.23	1.05–1.45	1.19	1.06–1.32
CyA/FK506	1.45	1.07–1.96	1.39	1.09–1.79

Abbreviations: OR, odds ratio; 95% CI, 95% confidence intervals.
Results of primary analysis are in bold (6-month remission free from both oral agents). For each of the six models, the outcome is defined on the basis of the duration of the remission (6 and 12 months) and the drug that was withdrawn. All models take into account the correlation in the data due to repeated observations in the same subject (mixed models) and are adjusted for the number of rituximab cycles (see Materials and Methods). Disease duration, toxicity, sex, and baseline body weight did not have any effect. 'Both P and C' indicates remission free from both prednisone and calcineurin inhibitors. 'Prednisone' indicates remission free from prednisone, but not from calcineurin inhibitors; 'CyA/FK506' indicates remission free from calcineurin inhibitors but not from prednisone.

genotype frequencies between patients presenting different sensitivities to rituximab (prednisone and calcineurin inhibitor–free remission for 12 months) versus 320 controls showed a trend to a higher frequency of the C allele coding for Arg ($P = 0.059$) in those patients who successfully stopped both oral agents for 12 months (Supplementary Table S3 online). All other comparisons were not statistically significant (not shown).

CD20 (MS4A1) and SMPDL3-B (SMPDL3-B) variations. The search for variants/mutations in *MS4A1* (exon 4) gene coding for CD20 epitopes 170ANPS173 or 182YCYSI186 and in *SMPDL3-B* (epitope 156ELWKPW161) was negative.

Modulation of podocyte shape and biochemistry. See Supplementary Results online.

DISCUSSION

In this follow-up study, we enrolled 46 children with INS dependent on both steroids and calcineurin inhibitors. These children had been in remission for at least 1 year and failed any attempts to withdraw either oral agent. Participants received one to five rituximab treatments (104 in total) during a median follow-up of 3 years (range 1–5). We found that (1) the probability of prednisone and calcineurin inhibitor–free remission for 6 months was achieved in approximately half of the cases after rituximab treatment, with greater response rates for single oral agent–free remission and considerable proportions of children still in remission at 1 (20%) and 2 (10%) years; (2) median time to relapse in children who completed prednisone and calcineurin inhibitor treatment was between 5.6 and 7.3 months after the initial rituximab infusion and between 8.5 and 11.6 months after the second and subsequent infusions; (3) rituximab adverse effects were mild and limited; finally, (4) we found that successful response to rituximab was associated with older age at diagnosis and longer time to reconstitution of circulating CD20. We did not find any association between the probability of remission and variation in FcγR, CD20, and/or *SMPDL-3B* polymorphisms.
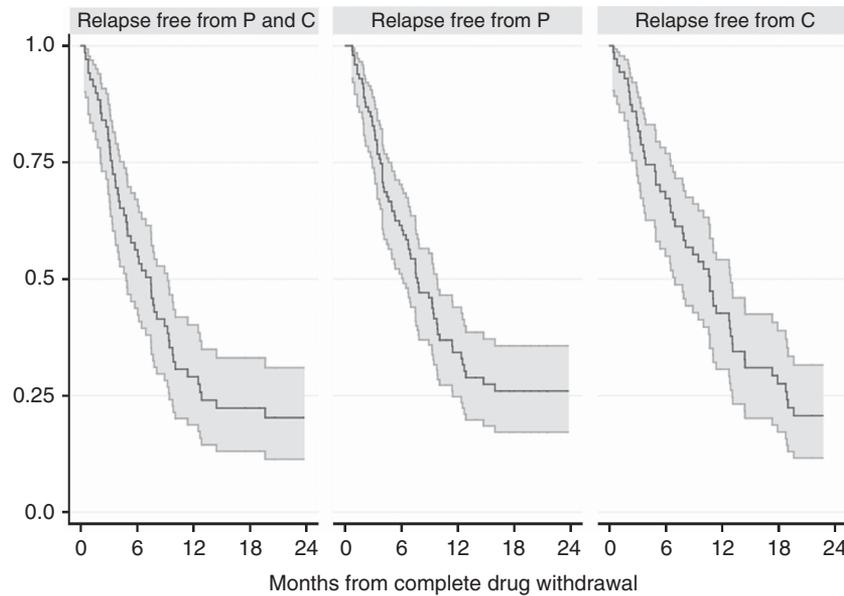
Figure 3 | Time to relapse in patients who completed prednisone and/or calcineurin inhibitor tapering and withdrawal. The three Kaplan–Meier survival curves (with 95% confidence intervals) estimate the time to relapse according to whether remission (absence of proteinuria) was maintained without prednisone and calcineurin inhibitors ('P and C'; left plot); avoiding prednisone ('P', with or without use of calcineurin inhibitors; middle plot); or avoiding calcineurin inhibitors ('C', with or without the use of prednisone; right plot).

Table 3 | Median relapse-free survival time (in months) in patients who completed prednisone and/or calcineurin-inhibitor tapering and withdrawal

	First course		Second and subsequent courses	
	Median	Interquartile range	Median	Interquartile range
Both P and C	5.6	4.3–8.1	8.5	6.5–11.7
Prednisone	6.5	4.8–9.6	10.9	8.1–15.6
CyA/FK506	7.3	5.6–10.2	11.6	8.9–15.8

Median times are the times at which half of the sample is still event free. Each of the three estimates is from a log-normal model where the outcome is defined based on drug that was successfully tapered and withdrawn (models are described in Table 3). 'Both P and C' indicates remission free from both prednisone and calcineurin inhibitors; 'prednisone' indicates remission free from prednisone, but not from calcineurin inhibitors; and 'CyA/FK506' indicates remission free from calcineurin inhibitors but not from prednisone.

The implications of our findings are fourfold. First, rituximab can be used to maintain remission for 6–12 months without prednisone and calcineurin inhibitors in ~50% of children with INS. As previous toxicity does not affect response to treatment, rituximab may represent an attractive alternative therapy to spare steroids and calcineurin inhibitors in the long term in these children. Second, relapse-free survival ranged from 6 months to 1 year, and this time interval may guide decision-making to personalize long-term treatment based on repeated rituximab infusions. As relapse-free intervals are longer after repeated infusions, additional treatments of frequent relapses may be justified provided that tapering of oral agents is successful. Third, age at diagnosis was strongly associated with better outcomes. Although age is

not a modifiable factor, it carries important prognostic information to plan long-term care, guide additional diagnostic testing, and inform future research including studies on mechanisms of sensitivity to rituximab therapy. Fourth, our study suggests that rituximab, as other immunosuppressive agents, may successfully control but does not cure the disease.

We did not observe severe side effects. Risks related to discomfort during rituximab infusion can be minimized with pretreatment and by slowing the infusion rate. We observed only few episodes of acute arthritis and no rituximab-associated lung injury, which is very rare and may not be related to rituximab considering that the unique patient with nephrotic syndrome who developed pulmonary fibrosis had respiratory symptoms before the first infusion.[19] The same seems true for other long-term complications including leukoencephalopathy,[20] considering that JC reactivation is not restricted to patients treated with rituximab,[21] and has never been reported in the case of isolated nephrotic syndrome. Considering the potential cost saving of one rituximab infusion as compared with a 6-month treatment with tacrolimus (see in Supplements a dedicated section on costs) and the low risks associated with the use of rituximab, including repeated infusions, equipoise may exist as to whether rituximab dependency may be less toxic than combined steroid and calcineurin-inhibitor dependency.

Failure to identify molecular predictors of sensitivity to rituximab and the tendency of INS to maintain some form of treatment dependency (i.e., either on oral drugs or rituximab) opens the question of how best and in whom rituximab can be used today. Our study suggests that rituximab may be used as a steroid and calcineurin
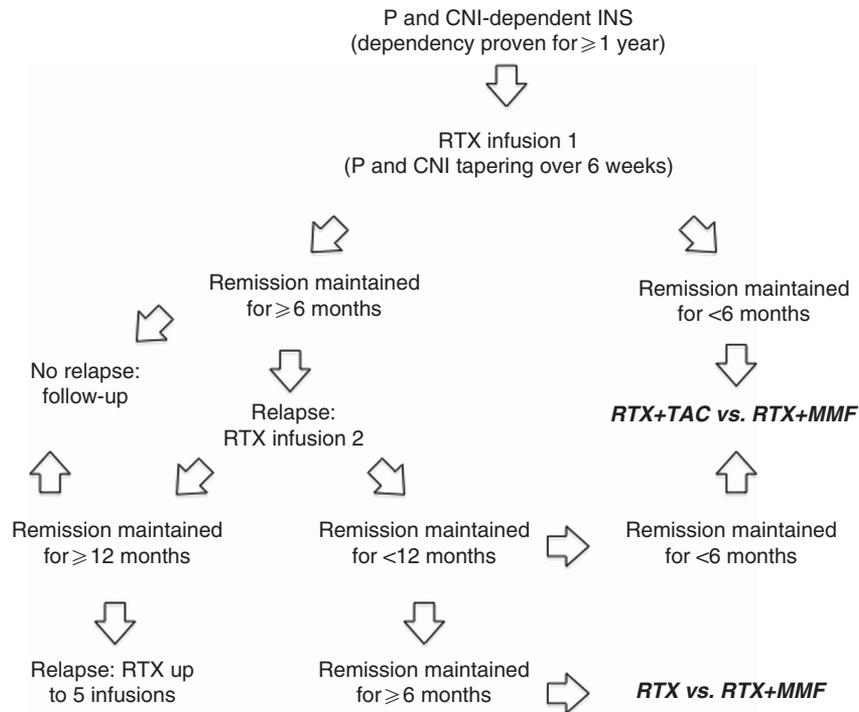
Figure 4 | Proposed strategies to maintain remission in prednisone (P) and calcineurin inhibitor–dependent idiopathic nephrotic syndrome. Bold indicates comparisons currently being tested in clinical trials (EudraCT number 2010-020184-20). CNI, calcineurin inhibitors; INS, idiopathic nephrotic syndrome; MMF, mycophenolate mofetil; RTX, rituximab; TAC, tacrolimus.

inhibitor–sparing agent in children in whom oral agents have been successful for at least 1 year and in whom, however, any attempts to taper them has failed. Depending on the duration of oral agent–free remission following rituximab infusion, different strategies can be considered (Figure 4). Our data indicate that attaining an oral agent–free remission for at least 6 months after the first rituximab supports sensitivity to rituximab therapy and potentially better response to subsequent infusions. However, longer remission times are the goal of the second and subsequent infusions. In case of long-lasting remission (>12 months), relapses may be treated with additional rituximab pulses, although safety data on repeated rituximab infusions beyond five courses are not available. If remission time is shorter, new strategies may be considered, including approaches currently being tested in clinical trials (Figure 4).

Time from rituximab-induced CD20 depletion to CD20 reconstitution may reflect the degree of immunosuppression and mirror sensitivity to rituximab. CD20 depletion involves mechanisms including apoptosis and complement- or antibody-dependent cell cytotoxicity mediated by Fcγ receptors in natural killer cells.[22] We were unable to support an association between functionally relevant polymorphisms (including *FCGR3A* rs396991 and *FCGR2A* rs1801274 SNPs encoding the natural killer cell FcγRIII protein) and rituximab activity that have been identified in patients with follicular lymphoma.[23–26] Similarly, our data do not support the effect of other potential modifiers of rituximab activity

including mutations in exon 4 of *MS4A1* coding for CD20-binding sites (extracellular loops Rp15-C9 and Rp5-L)[27] and in *SMPDL3-B* coding for sphingomyelin phosphodiesterase acid–like-3b,[18,28,29] an additional target of rituximab in human podocytes. Finally, our experimental data did not reveal any difference in the degree of actin cytoskeleton remodeling and suPAR-dependent signaling observed in normal human podocytes cultured in the presence of sera from patients with different clinical responses to rituximab. In contrast to what we observed in recurrent focal segmental glomerulosclerosis after transplantation,[18] rituximab administration *in vitro* did not prevent actin cytoskeleton remodeling observed after exposure to patient sera. This apparent discrepancy may be due to the more benign course in this study population, in whom circulating suPAR levels were similar to age-matched normal controls. These negative findings suggest that in this patient population systemic rather than direct effects on the podocyte may mediate the response to rituximab.

Although our study is the first longitudinal cohort study of rituximab in INS, it has limitations: it is not a clinical trial, the population enrolled is of limited size and data were collected in a single tertiary referral center. Further data from international collaborations are necessary to confirm our results. To minimize bias, we followed up patients prospectively using the infrastructure of a clinical trial including verification of eligibility criteria for study entry, standardization of interventional drug delivery, outcome assessment, and

safety monitoring.[17] These elements make our study difficult to compare with current literature data, which are based on uncontrolled case series or retrospective studies of heterogenous populations,[14–16] including patients who were dependent or resistant to a single drug (in general steroids), and cases with variable sensitivity to an association of steroids and calcineurin inhibitors. In addition, no studies on pharmacogenomics of rituximab have been conducted so far. Additional data from more homogenous patient populations are needed.

In summary, rituximab therapy can be safely and repeatedly used to attain prednisone and calcineurin inhibitor–free remission in a considerable proportion of children with INS dependent on both these agents. Remission can be achieved for 6 months in half of these children, with longer remission times following repeated treatments, greater response rates for single oral agent–free remission, and better outcomes in older children. However, in a similar proportion of cases, rituximab is ineffective or may allow limited steroid or calcineurin-inhibitor sparing. Further research is needed to understand the mechanism of action of rituximab in INS and to identify patients who will benefit most from rituximab therapy. Although rituximab represents a potential steroid and calcineurin inhibitor–sparing agent in several children with dependent forms of INS, rituximab alone does not seem to cure this disease. Our study suggests that remission of nephrotic syndrome tends to remain dependent on some form of treatment, and highlights how far behind we are in the management of this disease.

MATERIALS AND METHODS
Design overview
Half of the children who participated in a previous clinical trial[17] (13 children originally assigned to the intervention group and 14 originally assigned to the control group who received rituximab at the end of the trial) gave consent for long-term follow-up. Children who were followed up outside the coordinating center were excluded ($n = 27$). Nineteen additional children, who were consecutively referred for INS after the completion of the trial, were eligible for the present study.[17] To be enrolled, children had to be 16 years old or younger, with an estimated creatinine clearance >60 ml/min per $1.73\,m^2$. They had to have a history of INS responsive to, and dependent on, both prednisone and calcineurin inhibitors for at least 1 year with remission (proteinuria $<4\,mg/m^2/h$) maintained with both oral agents. At least three attempts to withdraw oral agents had to be unsuccessful with disease relapse within 1–2 weeks of oral-agent withdrawal. Criteria for diagnosis of INS were the same as in the published trial.[17] Briefly, INS was diagnosed in the presence of nephrotic-range proteinuria ($>40\,mg/m^2/h$ or $>1\,g/day/m^2$, or protein to creatinine ratio (P/C) >4 in a single urine specimen) or between 5 and $40\,mg/m^2/h$ associated with hypoalbuminemia or dyslipidemia. According to our protocol, no renal biopsy was done because of clear drug sensitivity. Criteria for exclusion were infantile onset (<1 year), previous history of macro-hematuria, hepatitis B, hepatitis C, or HIV infection, positivity of any marker of autoimmunity (ANA, nDNA, ANCA), low C3 levels, and need of diuretics, albumin, or anticoagulant therapy. Steroid dependency was defined as responsiveness to full doses of prednisone (2 mg/kg)

and calcineurin inhibitors (5 mg/kg), with two consecutive relapses occurring during prednisone tapering or within 2 weeks of prednisone withdrawal. Toxicity was defined based on previously published criteria[17] (Supplementary Table S1 online). Our study has been approved by the local Ethical Committee of the Institute Giannina Gaslini and was registered in the European Registry EudraCT (EudraCT registration numbers 2007-007796-16; 2008-004486-26).

Healthy controls. A total of 320 healthy controls were genotyped as controls for *FCGR2B* and *FCGR3A* polymorphism studies. All the controls were Caucasian of Italian origin.

Laboratory investigations. See Supplementary Material online.

Initial rituximab infusion and treatment of relapses. All participants received an intervention schedule based on rituximab infusion and tapering of prednisone and calcineurin inhibitors. Tapering was done following the scheme below that allows drug withdrawal within 45 days. Rituximab (Mabthera; $375\,mg/m^2$ intravenous) was given intravenously once (in the absence of clinical signs of toxicity from steroids and/or cyclosporine) or twice (after 2 weeks from the first infusion in the presence of toxicity). The medication was diluted in normal saline (1 mg/ml) and administered at increasing speeds (0.5–1.5 ml/min) over ~ 6 h. The infusion was preceded by 2.5–5 mg of intravenous chlorphenamine maleate, 2 mg/kg methyl prednisolone in normal saline, and 8 mg/kg of oral paracetamol. Starting at 15 days, prednisone was tapered off by 0.33 mg/kg/week if proteinuria was <1 g/day. After 1 week, calcineurin inhibitors were also decreased by 50% and withdrawn after 4 additional days. In the case of relapse of proteinuria, a dose of 1 mg/kg prednisone was started and continued until stable remission. After 1 week, a single dose of rituximab ($375\,mg/m^2$ intravenous) was repeated, and prednisone tapered in the same way. In the case of persistence of proteinuria, or of a new recurrence within 5 months, cyclosporine was added at the starting dose of 4 mg/kg for 6 months, followed by reduction to 3 mg/kg. Rituximab infusion was repeated in the case of relapse (proteinuria $>40\,mg/m^2/h$ or $>1\,g/day/m^2$, or P/C >4 in a single urine specimen or between 5 and $40\,mg/m^2/h$ associated with hypoalbuminemia or dyslipidemia). Angiotensin-converting enzyme inhibitors or angiotensin receptor blockers were used at the discretion of the investigators, but kept constant during the study.

Study hypothesis, follow-up data, and outcomes
Hypothesis. We hypothesized that age (main exposure) and other patient characteristics, including gender, toxicity status, and disease duration, may affect the effects of rituximab. Sensitivity to rituximab linked to variation in FcγR, CD20, and/or *SMPDL-3B* may have addictive effects.[30,31]

Follow-up data. Children were seen every 3 months by a nephrologist, and as many times as necessary in case of complications or relapses. Study coordinators maintained ongoing contact with children and families to monitor clinical status and report potential adverse events. Routine clinical examinations (including white blood cell count) were performed monthly in the first 3 months, and then every 3 months. Blood pressure was measured at regular intervals (two times per week) by the family pediatrician who contacted the referent nephrologist for data reporting and therapy update. Urine protein excretion was measured weekly on 24-h urine collection, or more often in case of dipstick positivity. Determination of proteinuria at baseline and after 3 months was performed at a central laboratory. Dipstick for proteinuria

determination was evaluated daily. In case of dipstick positivity, the presence of proteinuria was confirmed with 24-h urine collection. Kidney function, plasma proteins, and cholesterol were obtained monthly. Counts of white blood cells and lymphocyte populations were monitored monthly.

Outcomes. The primary efficacy measure was the probability of maintaining remission (absence of proteinuria) for at least 6 months after successful tapering and complete withdrawal of prednisone and calcineurin inhibitors after the first rituximab infusion. Time was measured from the date of complete prednisone and calcineurin-inhibitor withdrawal (Figure 1). Remission for 12 months was also studied. We also studied single oral agent–free remission for 6 and 12 months. Secondary outcome was the relapse-free survival (time to relapse) from complete drug withdrawal (tapering failures were excluded). Relapse was diagnosed in the presence of proteinuria (see criteria for INS above) or restart of prednisone or calcineurin inhibitors to maintain remission. A data safety monitoring board reviewed the safety data of the present study. Protocol and consent documents were approved by the ethics committee of Giannina Gaslini Hospital.

Statistical analysis

We compared quantitative and qualitative variables with t-test and χ^2 method.

Probability of remission. We used mixed logistic models to study the relationship between age and the probability of remission for 6 and 12 months. Patient identifier was treated as a random factor to account the correlation of repeated measures in the same subject (i.e., each subject could receive several rituximab infusions and fail several times during follow-up). In the analysis of the first treatment, mixed modeling reduced to ordinary logistic regression.

Risk of relapse. We used parametric models for repeated survival times to study the relationship between age and the risk of disease relapse in children who completed tapering and oral-agent withdrawal after rituximab infusion. Relapse was defined as the restart of either or both steroids and calcineurin inhibitors to maintain remission. As to the remission analysis, rituximab could be repeated several times, and the same child could return at risk after each rituximab infusion if oral-agent tapering was successful. We censored observations at the study end date (25 September 2012) if children were still in remission, or when they left the study. We studied how the hazard function changed over time by plotting nonparametric (smoothed) estimates of this function. We also estimated the crude hazard rates for time intervals 0–1, 1–3, 3–6, 6–12, and >12 months. We selected a reasonable set of parametric survival models from the three-parameter γ-family by comparing observed rates within time intervals with model predictions and found that the log-normal model provided the best fit. We compared parametric survival models using formal tests (including testing the parameters of gamma nested models) and graphical assessment of the goodness of fit. We accounted for the correlation in the data due to recurrences and repeated treatments in the same patient using robust variance.

Modeling strategy. For both analyses, the initial model included age (exposure), sex, body weight, previous toxicity, and disease duration. The number of infusions was categorized as 1 and 2 or more (outcomes in 2 and 3 or more were similar). Previous randomization group in participants who were part of the clinical trial was tested as an effect modifier. We considered overall model fit and graphical tests based on residuals. Variations of the exposure regression coefficient were monitored while removing nonsignificant variables manually. Analyses were performed using STATA (www.stata.com).

AUTHORS CONTRIBUTIONS
GMG and JR designed the study, take responsibility for the integrity of the data and the accuracy of the data analysis, and drafted the paper. CS, A Ponticelli, and AM coordinated follow-up data collection and ensured the quality of data entry. All authors provided critical inputs for important intellectual content and final approval. GMG obtained funding and had the final responsibility to submit the manuscript for publication. Data safety and monitoring board members included Antonella Trivelli (MD), Giovanni Candiano (PhD), and Giorgio Piaggio (MD). SM-G and AF performed and analyzed the *in vitro* experiments.

SUPPLEMENTARY MATERIAL
Supplementary Information. Use of Rituximab in Children with Idiopathic Nephrotic Syndrome Dependent on Prednisone and Calcineurin-Inhibitors.
Table S1. Criteria for toxicity to standard medications.
Table S2. Effect of age (per year) on relapse free survival in children who completed drug withdrawal.
Table S3. Comparison allelic and genotype frequencies relative to FCGR2A rs1801274 and FCGR3A rs396991 SNPs between patients presenting different sensitivity to rituximab versus controls showed a trend to a higher frequency of the C allele coding for Arg ($P = 0.0592$) in those patients who successfully achieved withdrawal of both steroids and cyclosporine for 6.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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