80 mg per day) for 2 weeks, then 30 mg/m² on alternate days (maximum 40 mg per day) for 2 weeks, and then 15 mg/m² on alternate days (maximum 20 mg per day) for 2 weeks. When patients had relapses during the study period (1 year of follow-up), they received 60 mg/m² oral prednisolone three times a day (maximum 80 mg per day) until 3 days after complete remission was obtained, when tapering began. If patients were receiving ciclosporin at screening, tapering of this drug began at day 85 (patients received their first dose of rituximab or placebo on day 1), with discontinuation by day 169 (figure 1). If patients were taking any other immunosuppressive drugs, these drugs were discontinued by day 85 (figure 1).

Patients were followed up for 1 year (figure 1). Study visits occurred at baseline; at weeks 1, 2, 3, and 4; and every 4 weeks from week 5. Patients were deemed to have treatment failure if a relapse had occurred by day 85, FRNS or SDNS was diagnosed between days 86 and 365, or steroid resistance was noted (figure 1, appendix). We designed the study protocol with consideration for the placebo group as much as possible. When patients had treatment failure, their allocation code was urgently disclosed. If a patient with treatment failure was in the placebo group, he or she could then choose to begin the treatment deemed the best by investigators—eg, new immunosuppressive drugs—and continue in our study, or to enter a separate rituximab pharmacokinetic study after discontinuation or completion of our trial.

Outcomes

The primary endpoint was the relapse-free period (time of randomisation to the time of first relapse after starting the study treatment). The prespecified secondary endpoints were time to treatment failure, relapse rate (number of relapses per person-year), time to four relapses of nephrotic syndrome in the study period, time to two relapses during reduction of steroid treatment or within 2 weeks of discontinuation of steroid treatment, time to transition to steroid resistance, steroid dose after randomisation, changes in steroid dose before and after randomisation, peripheral blood B-cell count, peripheral blood B-cell depletion period, human antichimeric antibody production rate, and rituximab blood concentration. Safety endpoints were frequency and severity of adverse events, and abnormal values in biochemical tests and haematology assessments. We did post-hoc analyses of the effects of age at time of treatment and age at disease onset on median relapse-free period, the effect of concomitant angiotensin-converting-enzyme inhibitors and angiotensin-receptor blockers on median relapse-free period, time to FRNS or SDNS, the proportion of patients who could discontinue steroid treatment after study drug infusion, the time between cessation of steroid treatment and first relapse, the frequency of infections that required treatment, the effect of B-cell depletion on infections that required treatment and relapses, and changes in characteristics between baseline and 1 year.

Statistical analysis

On the basis of previous reports,[12,13,18] we assumed that 40% of the patients in the rituximab group and 10% of the patients in the placebo group would maintain remission 6 months after registration. 30 patients in each group would be needed to establish the superiority of the test treatment for the primary endpoint with 90% power at a 2·5% one-sided significance level under the assumption of exponential distribution of relapse-free survival time and proportionality of hazards.

We used the log-rank test to analyse the primary endpoint and other time-to-event endpoints. We did an interim analysis (appendix) after 30 patients had relapsed, with a significance level set at 0·25% (one-sided). We summarised time-to-event data with the Kaplan-Meier method and estimated therapeutic effect hazard ratios (HRs) and their 95% CIs with Cox regression.

We made no multiplicity adjustment in the analysis of secondary endpoints. We set the significance level at 5% (two-sided) and report two-sided p values. We calculated the relapse rate and the frequency of infection with the number of events per person-years. We compared groups with the computer-based permutation test, and calculated

For the **trial protocol** see http://www.med.kobe-u.ac.jp/pediat/pdf/rcrn01.pdf

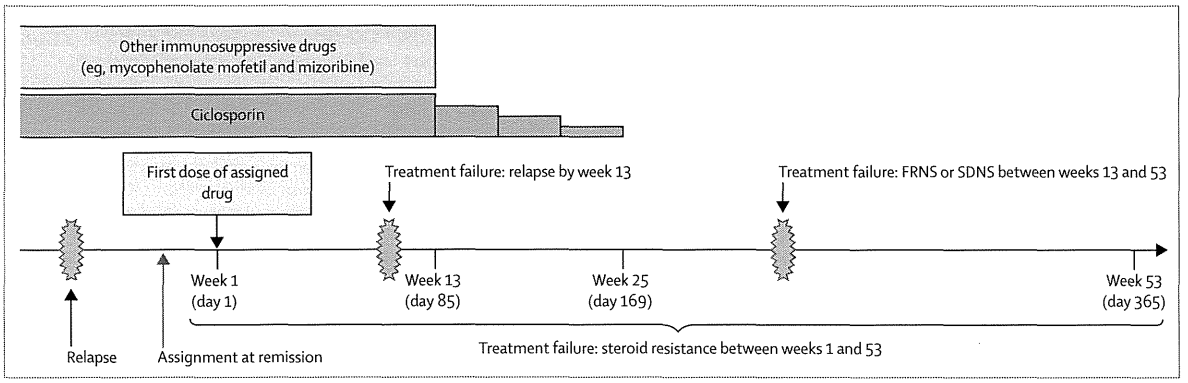

Figure 1: **Study design**
FRNS=frequently relapsing nephrotic syndrome. SDNS=steroid-dependent nephrotic syndrome.

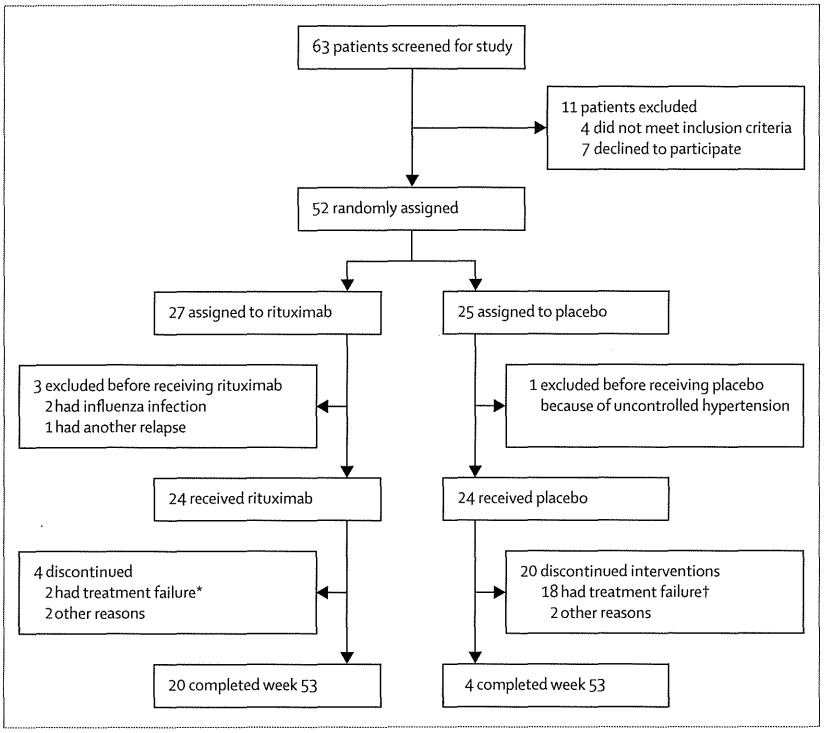

Figure 2: **Trial profile**
*One patient relapsed by week 13, and one was diagnosed with steroid resistance. †Ten relapsed by week 13, and eight were diagnosed with frequently relapsing nephrotic syndrome or steroid-dependent nephrotic syndrome after week 13.

the 95% CI of rate ratios fitting the negative binomial distribution and taking account of overdispersion. With the Wilcoxon rank-sum test, we compared daily steroid doses after randomisation and steroid doses before and after randomisation in both groups. We used the Kaplan-Meier method to assess the proportion of patients with human antichimeric antibody. Analyses were by modified intention to treat, including patients who received their assigned intervention. All analyses were done in SAS (version 9.1).

This trial is registered with the University Hospital Medical Information Network clinical trial registry, number UMIN000001405.

Role of the funding source

The funder of the study had no role in study design, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Between Nov 13, 2008, and May 19, 2010, 52 patients were randomly assigned to rituximab or placebo. Follow-up ended on Nov 10, 2011. The preplanned interim analysis showed that rituximab was superior to placebo, after which the independent data and safety monitoring committee advised us to discontinue randomisation as specified in the protocol. Therefore, randomisation ended earlier than planned, on May 21, 2010.

52 patients underwent randomisation (figure 2). 48 patients received the assigned intervention (figure 2) and were included in analyses. 20 patients given rituximab and 23 given placebo received all four doses. No patient dropped out before the first relapse. All 20 patients with treatment failure in the placebo group were enrolled into a separate rituximab pharmacokinetic study after discontinuation (n=18) or completion (n=2) of this trial.

Baseline characteristics in the two groups were similar (table 2). The predominant histological type in both groups was minimal change nephrotic syndrome (table 2). All patients were given steroids or immunosuppressants, or both, at relapse immediately before assignment (table 2). More than 70% of patients in both groups reported side-effects of steroid treatment (table 2).

By the end of 1 year of follow-up, 17 patients in the rituximab group and 23 in the placebo group had relapsed. The median relapse-free period was significantly longer in the rituximab group (267 days, 95% CI 223–374) than in the placebo group (101 days, 70–155; HR 0·27, 95% CI 0·14–0·53; p<0·0001; figure 3A). Post-hoc analyses showed that age at disease onset and age at time of treatment did not affect the median relapse-free period in the rituximab group (appendix). Concomitant angiotensin-converting-enzyme inhibitors or angiotensin-receptor blockers, or both, decreased the median relapse-free period in the rituximab group, although the difference was marginally significant (appendix).

Treatment failure was reported in ten patients in the rituximab group and 20 in the placebo group. The time to treatment failure was significantly longer in the rituximab group than in the placebo group (HR 0·27, 95% CI 0·12–0·59; p=0·0005; figure 3B). The relapse rate was significantly lower in the rituximab group (1·54 relapses per person-year [29 relapses in 18·81 person-years]) than in the placebo group (4·17 relapses per person-year [46 relapses in 11·03 person-years]; HR 0·37, 95% CI 0·23–0·59; p<0·0001). Only two patients in each group had frequent relapses in the study period. Time to two relapses during reduction of steroid treatment or within 2 weeks of discontinuation of steroid treatment was significantly longer in the rituximab group than in the placebo group (HR 0·19, 95% CI 0·07–0·54; p=0·0005). A post-hoc analysis showed that significantly more patients in the rituximab group did not experience frequent relapses or steroid dependence than in the placebo group (0·17, 0·06–0·46; p=0·0001; figure 3C). Two patients in the rituximab group had steroid-resistant relapses, compared with no patients in the placebo group.

Mean daily steroid dose after randomisation was significantly lower in the rituximab group than in the placebo group (9·12 mg/m² per day [SD 5·88] *vs* 20·85 mg/m² per day [9·28]; p<0·0001). Mean daily steroid (prednisolone) dose in the rituximab group

decreased significantly after randomisation, but did not change significantly in the placebo group (table 3). Exploratory analyses showed that the proportion of patients who could discontinue steroid treatment after the study drug infusion was similar in the rituximab group (21 of 24, 88%) and the placebo group (19 of 24, 79%; p=0·70). However, median time between cessation of steroid treatment and first relapse was significantly longer in the rituximab group (211 days, 95% CI 166–317) than in the placebo group (42 days, 14–98; HR 0·27, 95% CI 0·14–0·54; p<0·0001). The height-for-age *Z* score improved slightly 1 year after rituximab treatment compared with baseline, although the difference was not significant (appendix). Height *Z* score also seemed to improve in children with residual growth potential in the rituximab group, but again the difference was not significant (appendix).

Most adverse events were mild, and no patients died during the trial. Although more patients had serious adverse events in the rituximab group than in the placebo group (table 4), the difference was not significant (p=0·36). The most common grade 3–4 adverse events in the rituximab group were hypoproteinemia, lymphocytopenia, and neutropenia (table 5). Post-hoc analyses of adverse events showed that the incidence of infections that required treatment were similar in both groups (4·55 infections per person-year [105 infections in 23·08 person-years] *vs* 3·45 infections per person-year [42 infections in 12·18 person-years]; HR 1·27, 95% CI 0·77–2·07, p=0·21). More patients had mild infusion reactions in the rituximab group than in the placebo group (table 4), but the difference was not significant (p=0·12). No grade 3 or 4 infusion reactions were reported in either group (table 4).

The peripheral blood B-cell count decreased substantially immediately after the first dose of rituximab (figure 4), with a median period of B-cell depletion (<5 cells per μL) of 148 days (95% CI 131–170). B-cell counts returned to within the normal range in all patients given rituximab by day 253 (median 118 cells per μL, 95% CI 113–250). By contrast, peripheral blood B-cell count did not change in the placebo group (data not shown).

We did a post-hoc analysis of the effects of B-cell depeletion on relapses and infections. No relapses were reported in the rituximab group during the period of B-cell depletion. However, the rate of infections requiring treatment was higher during the B-cell depletion period (8·43 infections per person-year [49 infections in 5·81 person-years]) than outside of this period (3·24 infections per person-year [56 infections in 17·27 person-years]; HR 0·39, 95% CI 0·27–0·58; p<0·0001); although most were grade 1 respiratory-tract infections. The cumulative proportion of patients with human antichimeric antibody at day 365 was 14% (95% CI 5–38). Blood concentrations of rituximab are shown in table 6.

Discussion

We have shown that the relapse-free period increases with rituximab in patients with childhood-onset, complicated FRNS and SDNS. Adverse events were generally mild and the frequency of serious adverse

	Rituximab (n=24)	Placebo (n=24)
Age (years)	11·5 (5·0)	13·6 (6·9)
Duration of disease (years)	7·9 (4·7)	8·0 (5·4)
Sex		
Male	18 (75%)	16 (67%)
Female	6 (25%)	8 (33%)
Height (cm)	137·7 (21·4)	143·4 (20·4)
Height-for-age Z score	−0·96 (1·37)	−0·88 (1·26)
Weight (kg)	44·0 (18·6)	47·5 (15·6)
Body-mass index	22·3 (4·9)	22·6 (4·3)
Systolic blood pressure (mm Hg)	112·3 (11·0)	111·0 (9·6)
Diastolic blood pressure (mm Hg)	65·6 (9·9)	66·8 (8·2)
Serum creatinine (μmol/L)	39·78 (13·26)	44·20 (15·91)
Estimated glomerular filtration rate (mL/m per 1·73 m²)	128·9 (20·6)	126·4 (26·0)
Serum total protein (g/L)	58 (6)	59 (6)
Serum albumin (g/L)	34 (6)	34 (5)
Urinary protein to creatinine ratio (mg/mg)	0·13 (0·11)	0·11 (0·10)
Steroid and immunosuppressant use at relapse immediately before assignment		
Ciclosporin, mycophenolate mofetil, and daily steroids	1 (4%)	0
Ciclosporin, mizoribine, and daily steroids	3 (13%)	3 (13%)
Ciclosporin and daily steroids	0	1 (4%)
Mycophenolate mofetil and daily steroids	0	1 (4%)
Mizoribine and daily steroids	1 (4%)	1 (4%)
Daily steroids with no immunosuppressant	1 (4%)	0
Ciclosporin, mycophenolate mofetil, and steroids on alternate days	2 (8%)	0
Ciclosporin, mizoribine, and steroids on alternate days	6 (25%)	4 (17%)
Ciclosporin and steroids on alternate days	2 (8%)	5 (21%)
Mycophenolate mofetil and steroids on alternate days	0	0
Mizoribine and steroids on alternate days	3 (13%)	3 (13%)
Steroids on alternate days with no immunosuppressant	1 (4%)	2 (8%)
Ciclosporin and mycophenolate mofetil, with no steroids	0	0
Ciclosporin and mizoribine, with no steroids	1 (4%)	1 (4%)
Ciclosporin, with no steroids	1 (4%)	2 (8%)
Mycophenolate mofetil, with no steroids	0	0
Mizoribine, with no steroids	2 (8%)	1 (4%)
No steroids or immunosuppressant	0	0
Renal histology		
Minimal change	21 (88%)	23 (96%)
Focal segmental glomerulosclerosis	2 (8%)	1 (4%)
Unknown	1 (4%)	0
Steroid toxicity*	17 (71%)	19 (79%)
Time between relapse immediately before screening and previous relapse		
<180 days	15 (63%)	18 (75%)
≥180 days	9 (38%)	6 (25%)
Time from assignment to start of assigned intervention (days)	6·3 (2·7)	6·3 (3·4)

Data are mean (SD) or n (%). *Complications induced by steroid treatments, such as hypertension, short stature, diabetes, glaucoma, cataract, central obesity, and osteoporosis.

Table 2: **Baseline characteristics**

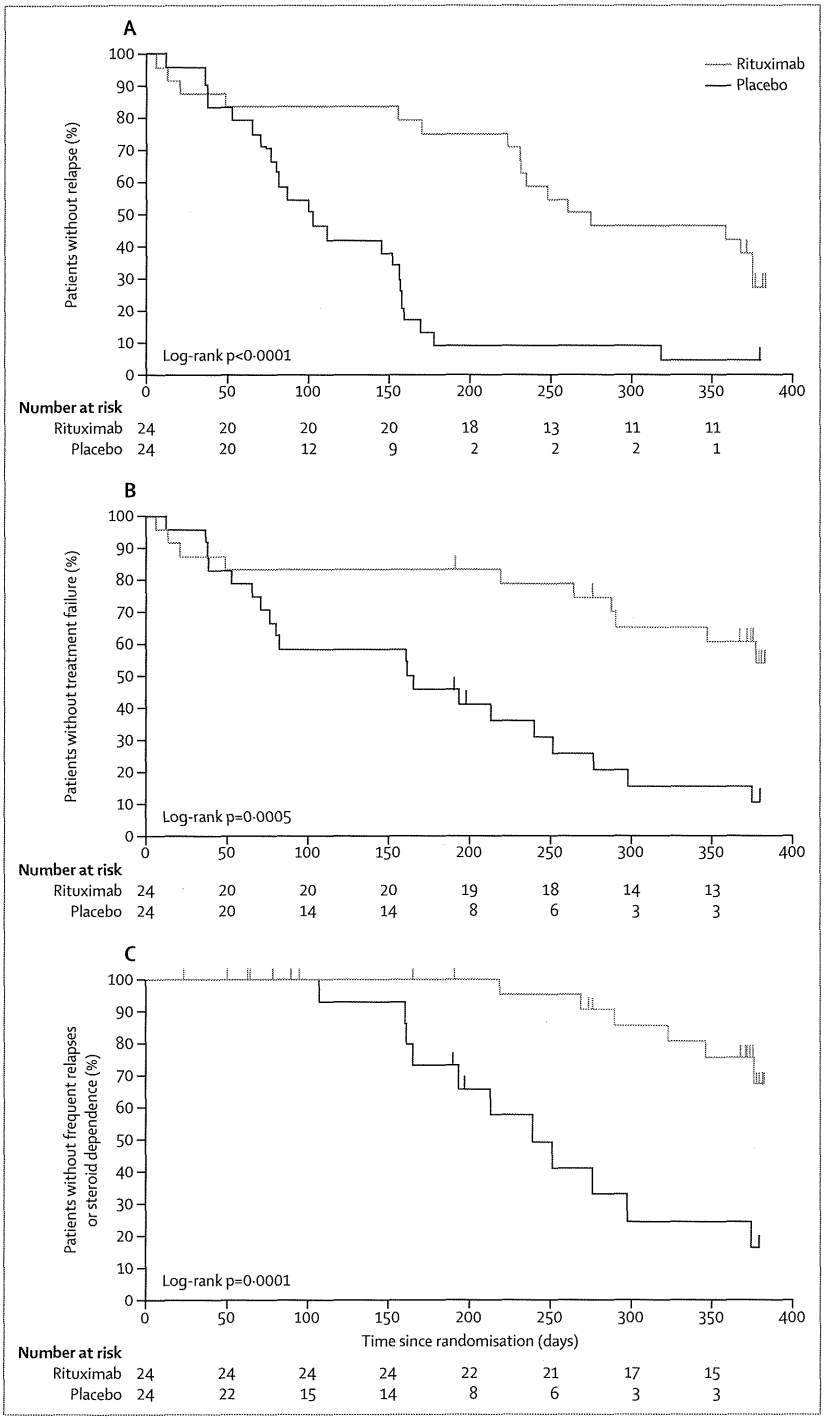

Figure 3: **Kaplan-Meier curves for primary and secondary outcomes**
(A) Patients without relapse. (B) Patients without treatment failure. (C) Patients without frequent relapses or steroid dependence. Vertical lines indicate censoring.

events did not differ significantly between groups. As far as we are aware, we are the first to show that rituximab is safe and effective for at least 1 year of treatment in a multicentre, double-blind, randomised, placebo-controlled trial (panel).

	Number of patients*	Daily prednisolone dose in the 365 days before randomisation (mg/m² per day)	Daily prednisolone dose after randomisation (mg/m² per day)	p value
Rituximab	19	19·13 (9·94)	8·37 (5·62)	<0·0001
Placebo	21	18·02 (10·15)	21·02 (9·81)	0·21

Data are mean (SD), unless otherwise stated. *Number of patients in each group for whom prednisolone doses were available for 365 days before randomisation.

Table 3: **Change in daily prednisolone dose before and after randomisation, by group**

	Rituximab (n=24)	Placebo (n=24)
Number of adverse events	357	251
Patients with ≥1 adverse event	24 (100%)	23 (96%)
Number of serious adverse events	16	7
Patients with ≥1 serious adverse event	10 (42%)	6 (25%)
Deaths	0	0
Number of grade 3 adverse events	24	15
Patients with ≥1 grade 3 adverse event	8 (33%)	3 (13%)
Number of grade 4 adverse events	3	0
Patients with ≥1 grade 4 adverse event	1 (4%)	0
Cases of infections that required treatment	105	42
Grade 1	1	0
Grade 2	101	42
Grade 3	3	0
Grade 4	0	0
Patients with ≥1 infection	23 (96%)	18 (75%)
Total number of infusion reactions	41	26
Grade 1	36	25
Grade 2	5	1
Grade 3	0	0
Grade 4	0	0
Patients with ≥1 infusion reaction	19 (79%)	13 (54%)

Data are n or n (%). Adverse events were categorised according to the National Cancer Institute's Common Terminology Criteria for Adverse Events (version 3.0).

Table 4: **Adverse events**

Patients with complicated FRNS or SDNS usually have a long history of the disease, and many of those included in our trial were receiving fairly high daily doses of steroids with or without immunosuppressive agents to prevent frequent relapses. Therefore, some patients did not meet the usual criteria for frequent relapses or steroid dependence before randomisation. However, more than 80% of patients in our study were treated with prednisolone at the relapse immediately before randomisation, and the mean daily prednisolone dose for 1 year before randomisation was about 20 mg/m². These facts indicate that overall disease activity was high. To allow enrolment of these patients into our trial, we modified the definitions of frequent relapses and steroid dependence immediately before the trial.

A fairly large study[16] of rituximab treatment for patients with steroid-dependent and calcineurin inhibitor-dependent idiopathic nephrotic syndrome, similar to those enrolled in our trial showed that the 6-month probability of remission after the first infusion was 48%. The relapse-free period was similar to that in our study, further emphasising the efficacy of the drug. Our finding that the age at disease onset and age at time of treatment did not greatly affect the outcome is fairly consistent with data from an uncontrolled study that also included adult patients.[19] The fact that patients in the rituximab group who were concomitantly treated with angiotensin-converting-enzyme inhibitors or angiotensin-receptor blockers, or both, had earlier relapses suggests that these drugs did not prevent relapses and patients treated with those drugs had more active disease.

More than half the patients in the rituximab group could discontinue steroids for more than 200 days without relapses after receiving rituximab. A long steroid-free period would allow patients to recover from side-effects, such as impaired growth. Indeed, the height *Z* score seemed to improve 1 year after treatment in the rituximab group, although the difference was not significant. Long-term follow-up studies are needed to clarify the effects of rituximab treatment for recovery from impaired growth.

Rituximab does not increase the frequency of infection when used to treat rheumatoid arthritis.[20,21] However, the rate of infections requiring treatment was higher during the period of B-cell depletion in the rituximab group in our study than when B cells were not depleted. Therefore, attention should be paid to infections during this phase, although most infections in our study were mild and treatable. In studies of patients with complicated nephrotic syndrome who had been taking rituximab, one child died because of pulmonary fibrosis[22] and another patient with fulminant myocarditis due to enterovirus underwent heart transplantation.[23] However, we recorded no deaths or cases of pulmonary fibrosis or myocarditis.

Although we recorded no relapses during B-cell depletion, a low B-cell count could offer clues about whether relapse is likely. Because our protocol did not specify that peripheral B-cell count should be established at time of relapse, a clear correlation between B-cell count and relapse could not be identified. We believe that continued monitoring of the B-cell count throughout the study period, especially at the time of relapse, will be necessary in future investigations. Another limitation of our study was the fairly short observation period. Therefore, the long-term prognosis of patients given rituximab is unclear. Specifically, we are aware of the possibility that not all rare and serious adverse effects were detected in our study—eg, progressive multifocal leukoencephalopathy is known to be a serious side-effect of rituximab.

All patients in our trial had relapsed by 19 months after randomisation. To extend the relapse-free period, further modification of the rituximab treatment and possibly adjunct immunosuppressive therapies might be necessary.

	Rituximab (n=24)		Placebo (n=24)	
	Grade 3	Grade 4	Grade 3	Grade 4
Gastritis	1 (4%)	0	0	0
Gastroenteritis	1 (4%)	0	0	0
Gum infection	1 (4%)	0	0	0
Cellulitis	1 (4%)	0	0	0
Hypertension	1 (4%)	0	0	0
Respiratory disturbance	1 (4%)	0	0	0
Acute kidney failure	1 (4%)	0	0	0
Haemorrhagic cystitis	1 (4%)	0	0	0
Hyperuricaemia	0	1 (4%)	0	0
Hypoproteinaemia*	6 (25%)	0	6 (25%)	0
Adrenal insufficiency	1 (4%)	0	0	0
Nettle rash	1 (4%)	0	0	0
Lymphocytopenia	4 (17%)	0	4	0
Neutropenia	2 (8%)	2 (8%)	0	0
Increased aspartate aminotransferase	0	0	1 (4%)	0
Increased alanine aminotransferase	1 (4%)	0	2 (8%)	0
Increased γ-glutamyl transpeptidase	0	0	1 (4%)	0
Increased creatine phosphokinase	1 (4%)	0	0	0
Hypophosphataemia	0	0	1 (4%)	0

Data are n (%). *Not known to be a side-effect of rituximab and was probably caused by the original disease rather than by rituximab or placebo, because occurred at time of relapse in both groups; other adverse events were known to be caused by the study drug.

Table 5: **Grade 3–4 adverse events**

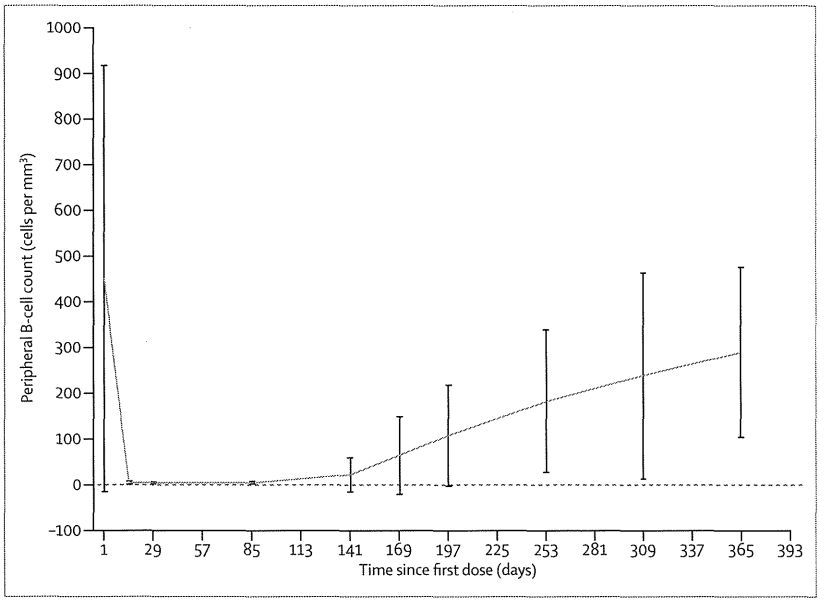

Figure 4: **Mean peripheral B-cell counts in the rituximab group**
Error bars show SD.

Additionally, a comparison of the efficacy, safety, and cost-effectiveness of various rituximab dosing regimens and B-cell-driven regimens still needs to be done.[24] An uncontrolled study[19] showed the importance of long-term follow-up after a core trial assessing the risk and benefit of rituximab treatment. We are preparing a retrospective

	Number of patients for whom data available	Mean rituximab blood concentration (ng/mL)
Day 1 (before the first infusion of rituximab)	24	0
Day 22 (before the fourth infusion of rituximab)	23	156 000 (53 700)
Day 85	24	28 800 (17 500)
Day 169	24	2320 (2680)
Day 365*	23	0

Data in parentheses are SD. *Three samples included here were not assessed on day 365; assessments occurred on days 189, 268, and 271, because these patients discontinued assigned treatment because of treatment failure. However, the values were less than the detectable range and so were included as data for day 365.

Table 6: Blood concentrations of rituximab

***Panel:* Research in context**

Systematic review

On completion of our trial, we did a systematic review to identify any randomised controlled trial in which the effectiveness or safety of rituximab, or both, was assessed in children with complicated frequently relapsing nephrotic syndrome (FRNS) or steroid-dependent nephrotic syndrome (SDNS). We searched Medline, Embase, and the Cochrane Library for reports published in any language before Oct 5, 2013, with terms such as "nephrotic syndrome", "rituximab", and "child" (appendix). We identified two open-label, randomised controlled trial (appendix). Meta-analyses of remission frequency at 3 and 6 months confirmed the effectiveness of rituximab in these children, and showed an increase in the remission rate of about 50% at 3 months and of more than 300% at 6 months (appendix).

Interpretation

As far as we are aware, ours is the first randomised, placebo-controlled clinical trial in which the efficacy and safety of rituximab for childhood-onset, complicated FRNS and SDNS have been assessed. Rituximab should be considered as an effective treatment for children with these disorders.

long-term follow-up study of patients enrolled in our trial, with a focus on clinical courses, treatments after the clinical trial, growth, and late adverse effects.

The exact pathogenesis of nephrotic syndrome is unclear, but T-cell-mediated immunological abnormalities are thought to have a role.[25] Several studies[26–29] have shown that B cells can promote T-cell activation, mediate antibody-independent autoimmune damage, and provide costimulatory molecules and cytokines, which sustain T-cell activation in autoimmune diseases. Rituximab inhibits B-cell proliferation and induces B-cell apoptosis.[30] This action leads to B-cell depletion and hence suppression of interactions between B cells and T cells, which could prevent recurrences of nephrotic syndrome. Impaired function of regulatory T cells in patients with minimal change nephrotic syndrome and induction of remission in nephrotic syndrome by regulatory T cells have been reported previously.[31–33] Rituximab could induce an increase in the number and function of regulatory T cells.[34] Rituximab-maintained remission in nephrotic syndrome could be due to the restoration of function of regulatory T cells. Fornoni and colleagues reported[35] that rituximab binds directly to an acid sphingomyelinase-like phosphodiesterase 3b on the cell surface of podocytes, stabilising podocyte structure and function, which could lead to the prevention of recurrent focal segmental glomerulosclerosis. Whether a similar mechanism works in complicated FRNS and SDNS remains to be established.

Contributors

KIi and MS were responsible for the study concept. KIi, MS, and NT designed and managed the study. KNo, KK, KM, KA, KNa, YOht, ST, RT, HK, KIs, and SI collected and interpreted data. YOha did statistical analysis. RM did the systematic review. All authors were members of the writing group and agreed on the content of the report, reviewed drafts, and approved the final version.

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A multicenter randomized trial indicates initial prednisolone treatment for childhood nephrotic syndrome for two months is not inferior to six-month treatment

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In this multicenter, open-label, randomized controlled trial, we determined whether 2-month prednisolone therapy for steroid-sensitive nephrotic syndrome was inferior or not to 6-month therapy despite significantly less steroid exposure. The primary end point was time from start of initial treatment to start of frequently relapsing nephrotic syndrome. The pre-specified non-inferiority margin was a hazard ratio of 1.3 with one-sided significance of 5%. We randomly assigned 255 children with an initial episode of steroid-sensitive nephrotic syndrome to either 2 - or 6-month treatment of which 246 were eligible for final analysis. The total prednisolone exposure counted both initial and relapse prednisolone treatment administered over 24 months. Median follow-up in months was 36.7 in the 2-month and 38.2 in the 6-month treatment group. Time to frequent relaps was similar in both groups; however, the median was reached only in the 6-month group (799 days). The hazard ratio was 0.86 (90% confidence interval, 0.64–1.16) and met the non-inferior margin. Time to first relapse was also similar in both groups: median day 242 (2-month) and 243 (6-month). Frequency and severity of adverse events were similar in both groups. Most adverse events were transient and occurred during initial or relapse therapy. Thus, 2 months of initial prednisolone therapy for steroid-sensitive nephrotic syndrome, despite less prednisolone exposure, is not inferior to 6 months of initial therapy in terms of time to onset of frequently relapsing nephrotic syndrome.

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Idiopathic nephrotic syndrome (NS) is a disorder affecting the kidneys that is mainly characterized by high excretion of protein in the urine. Pediatric idiopathic NS is understood to be the most common cause of primary glomerular diseases, and it frequently occurs in infants aged 2–6 years. Most patients are presumed to have minor glomerular abnormality. Cellular immunologic abnormalities are believed to contribute to the condition, although its pathology remains unknown. In Europe and the United States, two in 100,000 children will develop idiopathic NS in a single year.[1] An 8-week corticosteroid regimen is the standard initial treatment for children with idiopathic NS, as outlined by the International Study of Kidney Disease in Children (ISKDC).[2,3] Although corticosteroids induce the remission of proteinuria in more than 80% of children with idiopathic NS, ~60% undergo proteinuria relapse. Previous research has shown that a high number of children undergo frequent relapse, and corticosteroid toxicities occur after repeated therapy.[2,3] Although some controlled studies[4–7] and a meta-analysis[8] show that long-term corticosteroid treatment up to 7 months maximum leads to a longer sustained remission of NS than ISKDC-recommended administration, the optimum

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[10]See appendix.

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dose and duration of initial therapy are still unknown. A Cochrane review concluded that a well-designed and adequately powered randomized controlled trial is required to establish the optimum dose and duration of treatment.[8] The purpose of this study is to investigate whether 2 months of initial prednisolone therapy (ISKDC regimen) is not inferior to 6 months of initial therapy with an increasing cumulative dose, and to compare adverse events between treatment regimens.

RESULTS

Patient population

The study was conducted from September 6, 2007 until February 8, 2013. Figure 1 shows the trial profile. We assessed 255 patients from 90 hospitals (61 general, 7 children's, and 22 university hospitals) for eligibility. We randomly assigned 128 patients to the 2-month prednisolone group and 127 patients to the 6-month prednisolone group. We excluded nine patients from the analysis: six did not receive trial medication because of either early relapse after remission during the initial 4-week prednisolone treatment, or withdrawn consent, and three were excluded owing to a lack of participant data. Thus, we analyzed data for 246 patients. Median follow-up was 36.7 months in the 2-month group (interquartile range 27.8–46.4 months) and 38.2 months in the 6-month group (interquartile range 28.6–48.5 months). There was no difference in characteristics between the two groups (Table 1).

Primary end point

The primary end point was defined as the duration from start of initial treatment to diagnosis of frequently relapsing nephrotic syndrome (FRNS), or 'time to FRNS'. By the end of the 24-month intervention period, we observed 54 events in the 2-month group (comprising 46 FRNS [definition 1, 28; definition 2, 18], and 8 requiring immunosuppressant administration) and 58 events in the 6-month group (comprising 45 FRNS [definition 1, 23; definition 2, 22], and 13 requiring immunosuppressant administration). Twenty-one patients required immunosuppressants owing to steroid-dependent or steroid-resistant relapse. Times to FRNS were similar in both groups: however, the median duration of time to FRNS was reached only in the 6-month group (at 799 days). The hazard ratio (HR) was 0.86 (90% confidence interval (CI), 0.64–1.16; Figure 2), and noninferiority of the 2-month group was confirmed significantly, with an HR margin of 1.3 ($P=0.01$). Post-hoc analyses showed that age groups did not affect the median duration of time to FRNS. The HRs (95% CI) were 0.92 (0.59–1.45), 0.86 (0.41–1.84), and 0.74 (0.31–1.77) for the age groups 1–5 years, 6–10 years, and 11–15 years, respectively.

Secondary end points

Times to first relapse were similar in both groups: the median was 242 days and 243 days in the 2-month and 6-month treatment groups, respectively (HR = 0.97; 95% CI, 0.72–1.31; $P=0.86$; Figure 3). The number of relapses per person-year during the trial intervention period was 1.25 times in the 2-month group and 1.33 times in the 6-month group, and the ratio was 0.94 (95% CI, 0.71–1.22; $P=0.65$, Table 2). The median cumulative dose of prednisolone during the 2-year trial period in the 2-month group was also significantly lower than in the 6-month group (4621.9 [interquartile range = 2191.3–7472.5] vs. 6484.8 [interquartile range = 3701.0–9577.9], $P<0.001$).

Adverse events

Frequency and severity of adverse events were similar in both groups (Table 3). Most adverse events were transient and occurred during initial therapy or relapse therapy. In our study, steroid dependency did not greatly affect the occurrence of adverse events. Two patients in the 2-month group had severe adverse events requiring hospitalization. One patient discontinued because of acute kidney failure during

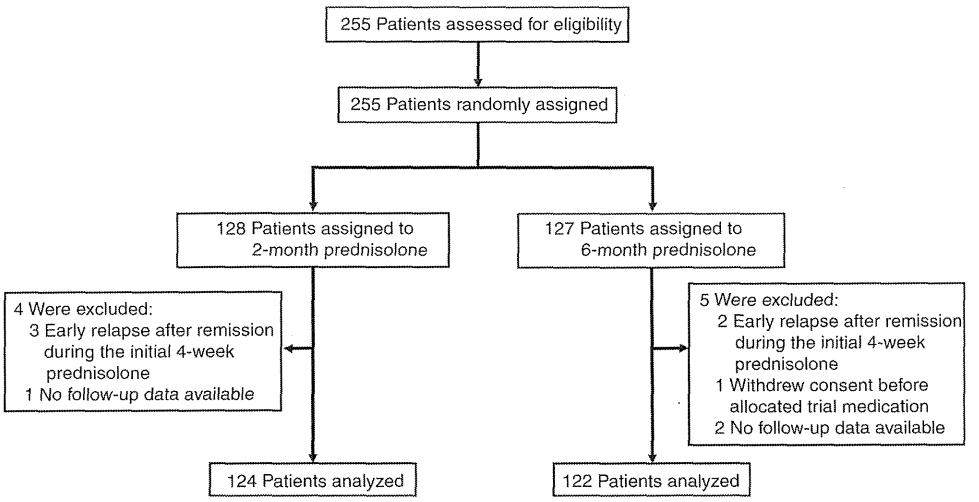

Figure 1 | Trial profile.

Table 1 | Baseline characteristics

	2-Month prednisolone ($n = 124$)	6-Month prednisolone ($n = 122$)	*P*-value
Male, *n* (%)	89 (71.8)	87 (71.3)	0.94
Age, mean (s.d.), years	6.7 (4.1)	6.3 (4.1)	0.42
Age group, years			
1–5, *n* (%)	67 (54.0)	66 (54.1)	0.99
6–10, *n* (%)	33 (26.6)	33 (27.1)	
11–15, *n* (%)	24 (19.4)	23 (18.9)	
Blood pressure, mean (s.d.), mm Hg			
Systolic	104.4 (10.7)	106.4 (12.0)	0.16
Diastolic	62.4 (10.0)	62.5 (11.3)	0.98
Serum albumin, mean (s.d.), g/l	1.4 (0.5)	1.4 (0.5)	0.90
Hospital, *n* (%)			
General	47 (65.3)	46 (64.8)	1.00
Children's	7 (9.7)	7 (9.9)	
University	18 (25.0)	18 (25.4)	
Quarterly distribution of disease onset, *n* (%)			
January–March	23 (18.7)	24 (19.7)	0.99
April–June	36 (29.3)	34 (27.9)	
July–September	30 (24.4)	31 (25.4)	
October–December	34 (27.6)	33 (27.0)	
Duration from the first episode to remission, mean (s.d.), days	9.7 (3.1)	10.0 (3.1)	0.45

Abbreviation: s.d., standard deviation.

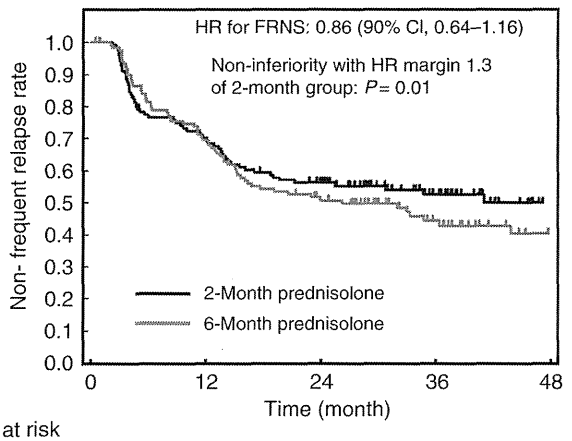

Figure 2 | Kaplan–Meier estimates of time to frequently relapsing nephrotic syndrome (FRNS). HR, hazard ratio.

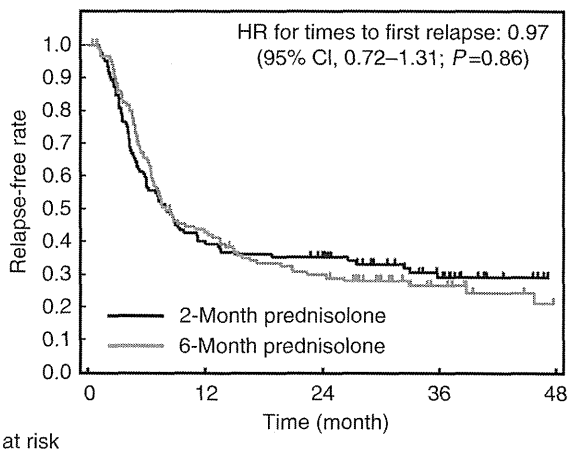

Figure 3 | Kaplan–Meier estimates of time to first relapse. HR, hazard ratio.

relapse (month 10) and recovered in 22 days. Another patient had pneumonia with influenza infection on the last date of the 2-month prednisolone treatment and recovered in 10 days. Height standard deviation scores show a significant decrease in growth at 2 months of follow-up compared with baseline ($P<0.003$). In both groups, this was restored within 9 months after initial treatment commenced. Notably, one patient in the 6-month group was diagnosed with possible adrenal insufficiency owing to steroid withdrawal according to clinical symptoms (mild headache and mild nausea) when the patient switched to trial medication after the initial dose of prednisolone. Symptoms disappeared on the same day of onset without further treatment.

DISCUSSION

Extension of initial steroid treatment for more than 3 months to decrease the risk of relapse in children with steroid-sensitive (SS) NS has been widely described in previous studies.[4–10] However, 80–90% of children diagnosed with SSNS who are given new corticosteroid treatments continue to relapse, and ~50% relapse frequently.[11] Therefore, initial approaches to SSNS therapy are likely to be substantially

Table 2 | Number of relapses

	Total number of relapses	Duration of observation (person-year)	The number of relapses (per person-year)	Ratio of the number of relapses (CI)	*P*-value
2-Month prednisolone	301	240.93	1.25	0.94	0.65
6-Month prednisolone	309	232.62	1.33	(0.71–1.22)	

Abbreviation: CI, confidence interval.

Table 3 | Adverse events during the 24-month trial intervention period[a]

Event	2-Month prednisolone $n = 124$	6-Month prednisolone $n = 122$	*P*-value
Hypertension[b]	15	9	0.24
Cushingoid appearance			
Cushing (moon face)[b]	54	61	0.46
Central obesity[b]	20	34	0.052
Striae	1	0	1.00
Adrenal insufficiency	0	1	1.00
Ophthalmological abnormalities			
Glaucoma[b]	19	13	0.31
Cataract	0	0	
Severe infections			
Pneumonia[c]	1	0	1.00
Peptic ulcer	1	0	1.00
Acute kidney failure[c]	1	0	1.00
Hyperglycemia	2	3	0.64
Increased laboratory data			
AST[b]	14	11	0.58
ALT[b]	26	16	0.14
Amylase	3	0	1.00

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase.
[a]Data are expressed as the number of events.
[b]Multiple reports were recorded for these adverse events.
[c]Severe adverse events requiring hospitalization.

different.[12] Our results demonstrate that extending initial steroid treatment, and even increasing the total dose of prednisolone (2240 vs. 3885 mg/m^2), does not improve clinical outcomes (time to FRNS, time to first relapse, the number of relapses, total prednisolone dose, and adverse events) for pediatric NS. Our results add to the Cochrane review by Hodgson *et al.*[8] by comparing treatment regimens of 2 vs. 6 months.

Our findings build on a 2013 study from the Netherlands. This well-designed, randomized, double-blind, placebo-controlled trial of children with NS clearly showed no improvement in clinical outcomes when the initial prednisolone treatment was extended from 3 to 6 months without an increasing cumulative dose.[13] However, despite the results of this trial, the most effective duration and dosage of prednisolone treatment for an initial episode of SSNS is still under debate.[14] Although our study has demonstrated that increasing the total dose in 2-month or 6-month regimens does not improve clinical outcomes, further investigation is still required to determine the most effective duration and dosage regimen for initial SSNS.

A key strength of our trial is its unique design. This is a multicenter, randomized, controlled trial for noninferiority that compares the efficacy of the 2-month ISKDC regimen with a 6-month, long-term prednisolone regimen. The most important clinical objective of initial treatment of SSNS is to prevent frequent relapses. Consequently, the primary end point was set to time to FRNS rather than initial relapses.

Our data from several analyses consistently support noninferiority of the ISKDC regimen. In the current trial, a HR threshold of 1.3 for noninferiority of the primary end point is slightly high given the feasible study size. However, the posterior probability that a HR would be smaller than 1.1 and 1.2 (HRs that are commonly used as an upper equivalence margin) was 91 and 97%, respectively.

In our trial, noninferiority was found in FRNS (primary end point) and first relapse (secondary end point) between the ISKDC and 6-month regimens. This means that many patients relapse even while taking tapering-dose prednisolone (Figure 3). Previous studies vary in their observation of (frequent) relapses from either the start or end of initial therapy.[8,13] However, if analyses are performed from the end of initial therapy, relapses during tapering-dose prednisolone cannot be counted, possibly resulting in an inadequate interpretation. Therefore, we selected observations primarily from the start of initial therapy. In our study design, steroid-dependent NS was predicted to occur more frequently because of its definition in the 6-month regimen. This is one reason why we selected FRNS as a primary end point, and not steroid-dependent NS. In fact, time to steroid-dependent NS was significantly higher in the ISKDC regimen group (data not shown).

No significant difference in adverse events was observed between the two regimens in our large-scale trial, which is a similar finding to previous small-scale trials.[4–7] Most adverse events were transient and occurred during initial or relapse therapy. However, because the ISKDC regimen is generally less likely to cause adverse events owing to the lower dosage and the shorter duration, its use can be recommended.

A limitation of our trial is the open-label design, which may have introduced preconception bias. However, as our trial design is a noninferiority trial with regular visits, and relapses are measured objectively, we cannot assume positive placebo effects. Therefore, the open-label design of this study may have limited impact on preconception bias. Moreover, the results of this trial may partially be due to the relatively high rate of relapse compared with other studies.[5] The high

rate of relapse may be owing to our definition of relapse (proteinuria 2+ or higher). It still remains unknown whether long prednisolone therapy consisting of a dose of 60 mg/m^2 per day for 6 weeks, followed by alternate-day doses of 40 mg/m^2 per 2 days for 6 weeks, is more effective against time to FRNS compared with the ISKDC regimen for treating idiopathic NS. In our study, only one patient withdrew consent. A low rate of consent withdrawal is common in Japan.[15,16] Cultural differences between countries may account for variations in rates of consent withdrawal.

In our study, steroid sensitivity was confirmed by day 21 in order to ensure time for eligibility screening. Generally, remission after 3 weeks is uncommon.[17] Therefore, as the effect of early confirmation of steroid sensitivity was slight, we decided to confirm steroid sensitivity by day 21.

We conducted a meta-analysis to address the differences between corticosteroid regimens in children with an initial episode of SSNS. We searched randomized controlled trials that compared durations of steroid therapy in children and reported the number of FRNS cases within 2 years (see the Supplementary Information online for a detailed search strategy). Meta-analysis of our study and five studies[6,13,18,19] showed a risk ratio (long vs short) of 0.99 (95% CI, 0.68–1.44, see Supplementary Figure 2A online), whereas meta-analysis of our study and the published studies only showed an risk ratio of 1.15 (95% CI: 0.95–1.40, Supplementary Figure 2B online). This result might indicate that long-term treatment is not superior but almost equivalent to ISKDC-standard therapy.

In conclusion, our study shows that extending initial prednisolone treatment from 2 to 6 months with an increasing dose does not improve clinical outcomes for pediatric NS. The original ISKDC regimen is not inferior to 6 months of initial therapy with an increasing cumulative dose. We assert that the ISKDC regimen is recommended as an initial treatment for pediatric idiopathic NS.

MATERIALS AND METHODS

Study design and patients

We conducted a multicenter, randomized, noninferiority, open-label trial at 90 hospitals in Japan and compared prednisolone treatment of 2 months (ISKDC regimen) with 6 months for children with a first episode of idiopathic NS. We diagnosed idiopathic NS and remission according to the ISKDC.[1] NS was defined as a urinary protein–creatinine ratio $\geqslant$1.8 and albumin levels $\leqslant$25 g/l in serum. Remission was defined as a negative dipstick analysis for 3 consecutive days. Patients aged 1–15 years with a first episode of idiopathic NS were eligible if they had remission within 3 weeks of prednisolone administration. Patients were ineligible if they had secondary NS, renal insufficiency defined as creatinine clearance of $\leqslant$60 ml/min per 1.73 m^2, active infections, poorly controlled hypertension, severe liver dysfunction, pregnancy, or a history of immunosuppressant administration.

Before enrollment, patients' guardians provided written informed consent, and informed assent was obtained from older children. This study was approved by the institutional review boards of participating hospitals, complied with the Declaration of Helsinki and the Declaration of Istanbul, and adhered to the International Conference on Harmonisation Guidelines on Good Clinical Practice.

Randomization

Patients were randomly assigned to either the 2-month or 6-month group in a 1:1 ratio at the Japan Clinical Research Support Unit. We applied a minimization method using a computer-generated sequence (SAS PROC PLAN) with age (1–10 years or 11–15 years), sex, and institution as adjustment (stratification) factors. Patients, patients' guardians, treating physicians, and individuals assessing outcomes and analyzing data were not blinded to the patients' treatment assignments. Apart from the trial statistician and the data-monitoring committee, all treating physicians and other investigators remained blinded to the trial results until follow-up was completed.

Procedures

The first patient was randomized in September 2007, and the last patient in January 2011. Follow-up started at diagnosis and was truncated when the last enrolled patients finished the 24-month intervention.

All patients diagnosed with a first episode of idiopathic NS started initial therapy of 60 mg/m^2 oral prednisolone in three divided doses (maximum of 80 mg/day) daily for 4 weeks. Patients underwent a screening examination and were registered after their eligibility, including remission, was verified. Participants switched to trial medication after initial doses of prednisolone were given (Figure 4). If participants relapsed after remission during the initial 4-week prednisolone treatment, they were excluded.

Trial medication consisted of initial treatment regimens and relapse treatment, and was completed within a total of 24 months in both groups (Figures 4 and 5). The duration of long-term prednisolone treatment was set to 6 months, which is consistent with recommendations from a non-Japanese randomized controlled trial.[8] The cumulative dose of initial treatment was 2240 mg/m^2 (2-month group) and 3885 mg/m^2 (6-month group). Participants

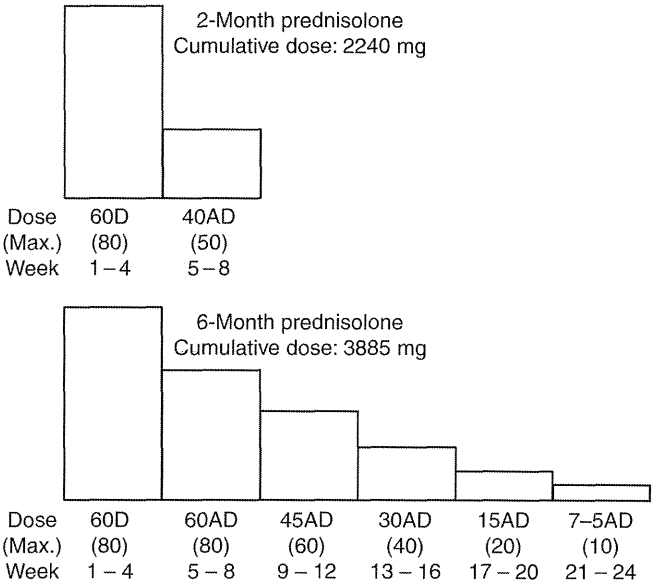

Figure 4 | Initial treatment regimens. Upper doses are in mg/m^2 per day. Maximum doses are in mg/day. D, daily; AD, alternate days.

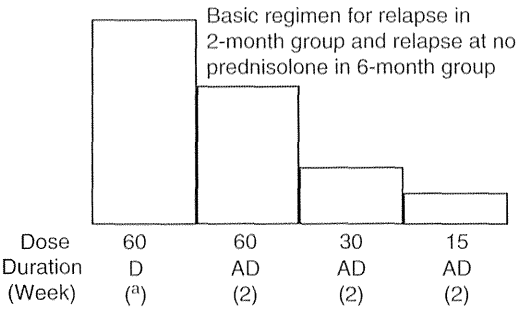

Figure 5 | Treatment regimens for relapse. [a]Until urinary protein is negative on 3 consecutive days using a urine dipstick test. Upper doses are in mg/m^2/day. A maximum dose of each is the same as initial treatments in Figure 4. AD, alternate days; D, daily.

who relapsed during the 24-month trial medication period received relapse treatment regimens (Figure 5). Relapse treatment was the same in both groups. However, relapse treatment given during the 6-month initial treatment was adjusted according to the initial treatment regimen in the 6-month group.

Participant characteristics (age, sex, physical characteristics, blood, and urine test results) were recorded at baseline. Clinical assessment and urine tests (urinalysis, qualitative and quantitative proteinuria, quantitative creatinine) were performed at 1, 2, 4, and 6 months after enrollment and every 3 months thereafter, and also on diagnosis of relapse. Blood analysis (complete blood cell count, blood chemistry) was performed at 1, 2, 6, and 12 months after enrollment, at the end of treatment, and at diagnosis of relapse only.

Adverse events were recorded throughout the trial period and assessed using Common Terminology Criteria for Adverse Events v3.0. Within 4 weeks of starting initial treatment, participants were screened for glaucoma by an ophthalmologist. Details about monitoring adverse events are described in the Supplementary Information online. Briefly, name of diagnosis, severity, seriousness, date of onset and disappearance, outcome, and assessment of causal relationship to the study drug were investigated at the start of study treatment and at 1, 2, 4, 6, 9, 12, 15, 18, 21, and 24 months after the start of study treatment (each within ± 2 weeks).

Outcomes

The primary end point was defined as the duration from start of initial treatment to diagnosis of FRNS, or 'time to FRNS'. Diagnosis of FRNS was based on the relapse dates according to the ISKDC. In our study, FRNS was defined as two relapses within 6 months of initial remission (definition 1), or four relapses within any 12-month period (definition 2), which included relapses during initial tapering treatment but excluded relapses with spontaneous remissions. Patients were observed for at least 2 years, on the basis of the Cochrane review, which states that the risk of relapse after 1–2 years was lower after long-term prednisolone treatment relative to the ISKDC regimen.[8] Relapse was defined as proteinuria 2+ or higher on dipstick analysis for 3 or more consecutive days or proteinuria 2+ or higher on dipstick analysis and serum albumin ≤ 25 g/l. Immunosuppressant administration was prohibited in the protocol. However, if administration was undertaken for clinical necessity, e.g., steroid dependency, it was treated as an event in the primary analysis. Data for patients who did not experience these events were considered censored at the last examination. Important secondary end points were time to first relapse, the number of relapses per person-year, total prednisolone dose, and adverse events.

Statistical analyses

The primary objective of this trial was to confirm noninferiority of initial therapy with 2-month treatment compared with 6-month treatment, with respect to time to FRNS. The noninferiority margin of HR for the 2-month to 6-month group was predefined to 1.3, and the significance level was set to 5% (one-sided). The former was determined based on surveys conducted among practicing pediatric nephrologists and other specialists before the protocol was developed.

On the basis of results from a previous study,[20] we assumed an event rate of 15 and 19% at 1 year in the 2- and 6-month groups, respectively. With a sample size of 125 patients in each treatment group, an HR test with a one-sided 5% significance level would have 70% power to confirm noninferiority. Accrual and follow-up times were specified to be 3 and 2 years, respectively.

As the previous study[20] was conducted more than 10 years earlier, it is possible that the current event rate of our study is lower than the previous study. We scheduled an interim analysis to take place just before the accrual completion date, which was performed in October 2010. A statistical test regarding the primary end point was not performed. The number of events observed matched that of the assumption, and the study plan was not changed.

Statistical analyses followed the protocol and the intention-to-treat principle. The Cox proportional hazard model was used to test noninferiority and estimate the HR with a 90% CI of the primary end point. The Kaplan–Meier method was used to summarize time to FRNS. These methods and the log-rank test were used to analyze time to first relapse. The number of relapses per person-year was calculated as the total number of relapses divided by the total observed person-years in each treatment group (Table 2). A permutation test was used to compare the number of relapses per person-year between groups. We compared the prednisolone total dose using the Wilcoxon test. The number of adverse events was compared using Poisson regression. For baseline characteristics, we compared distributions of continuous variables between groups using the *t*-test or Wilcoxon test, depending on the shape of the distribution. We analyzed categorical variables using the chi-squared test or Fisher's exact test. Posterior probability was calculated with the improper flat prior and the normal distribution to which log-HR was approximated. Except for noninferiority testing of the primary end point, we regarded a two-sided *P*-value < 0.05 to indicate statistical significance. We analyzed data using SAS software (version 9.3) and calculated the sample size using the SAS POWER procedure.

DISCLOSURE

This study was supported by a grant from the Ministry of Health, Labour and Welfare, Japan. All expenses were covered by the grant. NY has received grants from Novartis Pharma K.K. and Asahi Kasei Pharma Corporation and has also received lecture fees from Novartis Pharma K.K. and Asahi Kasei Pharma Corporation. KN has received lecture fees from Novartis Pharma K.K. and Asahi Kasei Pharma Corporation. KIs has received lecture fees from Novartis Pharma K.K. HH has received lecture fees from Asahi Kasei Pharma Corporation. MH has received lecture fees from Novartis Pharma K.K. and Asahi Kasei Pharma Corporation. SI has received lecture fees from Novartis Pharma and Asahi Kasei Pharma Corporation. YS has received lecture fees from Novartis Pharma K.K. HN owns stocks in Asahi Kasei Pharma Corporation. TI has received lecture fees from Takeda Pharmaceutical,

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SUPPLEMENTARY MATERIAL

Figure S2. (A) Meta-analysis of studies comparing 2–3 months of prednisolone to 5–6 months of prednisolone for children with their first episode of nephrotic syndrome, with an outcome showing the number of children with frequent relapses after 1–2 years. (B) Sensitivity analysis excluding Sharma 2002 (unpublished conference proceeding) from Fig. A.

Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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APPENDIX

Japanese Study Group of Kidney Disease in Children (JSKDC). JSKDC has been supported by grants from the Ministry of Health, Labour and Welfare, Japan. Members of the JSKDC are as follows.

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ORIGINAL ARTICLE

Rituximab-associated agranulocytosis in children with refractory idiopathic nephrotic syndrome: case series and review of literature

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ABSTRACT

Background. Agranulocytosis has been reported as a delayed-onset complication of rituximab treatment. However, the exact incidence and risk factors of this complication in patients with nephrotic syndrome remain unknown.

Methods. Records of 213 rituximab treatments for 114 patients with refractory nephrotic syndrome between February 2006 and April 2013 were reviewed to identify episodes of agranulocytosis (defined as an absolute neutrophil count of <500 mm^3).

Results. Eleven episodes of agranulocytosis were detected in 11 patients. Median time of onset of agranulocytosis was 66 days (range, 54–161 days) after rituximab treatment. Nine patients experienced acute infections and received antibiotics. All but one patient received granulocyte colony-stimulating factor. Agranulocytosis resolved in all cases within a median of 3 days. The incidence of agranulocytosis was 9.6% in total patients and 5.2% in all treatments. Median age of the 11 patients who developed agranulocytosis was 6.4 years at the first rituximab treatment, significantly younger than the median age of the 103 patients who did not (median, 12.5 years; $P = 0.0009$). Five patients received re-treatment with rituximab. No recurrence of agranulocytosis was observed in any patient.

Conclusions. It is important to pay extra attention to this clinically serious delayed-onset complication as it may be accompanied by life-threatening infections such as sepsis. Further clinical studies are needed to clarify its pathogenesis.

Keywords: agranulocytosis, B cell, infection, nephrotic syndrome, rituximab

INTRODUCTION

Rituximab is a chimeric monoclonal antibody directed against the cell surface antigen CD20 expressed on B lymphocytes. As

it has been proven to be effective in preventing relapses [1–7], it is increasingly being used in the treatment of patients with steroid-dependent nephrotic syndrome. While transient infusion-related side effects such as cough, sore throat, dyspnea and fever may occur, the toxicity of rituximab is relatively mild and well tolerated by most patients.

Nonetheless, agranulocytosis, a severe form neutropenia, has been reported to be a delayed-onset complication of rituximab treatment in patients with lymphoma [8–17] and autoimmune diseases [18–21]. Agranulocytosis usually occurs 1–6 months after rituximab administration and is often self-limiting. However, life-threatening infections can sometimes emerge. To date, there has been a case report of agranulocytosis associated with rituximab in nephrotic syndrome [22]. The exact incidence and risk factors of this complication in patients with nephrotic syndrome remain unknown. Herein, we analyzed all cases of agranulocytosis associated with rituximab treatment for refractory nephrotic syndrome in our center to evaluate the incidence and clinical characteristics of this complication.

MATERIALS AND METHODS

Records of 213 rituximab treatments administered between February 2006 and April 2013 for 114 patients with refractory nephrotic syndrome were reviewed to identify episodes of agranulocytosis, defined as Grade 4 neutropenia (an absolute neutrophil count of <500 mm^3) by the Common Terminology Criteria for Adverse Events (CTCAE) Ver. 4.0. Study protocol was based on the Declaration of Helsinki and was conducted with the approval of the off-label use of rituximab obtained from the ethical committee of our center (#645).

Indication of rituximab treatment was refractory steroid-dependent nephrotic syndrome (steroid dependence under immunosuppressive agents) or refractory steroid-resistant nephrotic syndrome (failure to go into remission despite a combination of cyclosporine and methylprednisolone pulse therapy). Rituximab was administered at a single dose of 375 mg/m^2 for 205 treatments, two doses of 375 mg/m^2 for one treatment (2-week interval for each infusion) and four doses of 375 mg/m^2 (once a week) for seven treatments. A total of 235 doses of rituximab were administered. To minimize infusion reactions, patients received intravenous methylprednisolone (1–1.5 mg/kg), oral acetaminophen (10 mg/kg, a maximum of 300 mg) and chlorpheniramine maleate (0.04 mg/kg, a maximum of 2 mg), 30 min prior to rituximab infusion. All patients were admitted to our center and were monitored for at least 24 h after rituximab treatment for infusion reactions. Complete blood counts and CD19+ B-cell counts were performed at least once a month until B-cell recovery. When a patient presented with fever at an emergency visit, procedures such as complete blood counts, C-reactive protein test, chest X-ray, urinalysis, blood and urine culture were immediately performed. Rituximab-associated agranulocytosis was defined as an episode during B-cell depletion where CD19+ B-cell count was <1% of total lymphocytes. B-cell recovery was defined as CD19+ B-cell count of equal to or >1% of total lymphocytes. Since January 2012, sulfamethoxazole/trimethoprim has been used at a dose of 5 mg/kg of trimethoprim once every 2 days for B-cell depletion with prophylaxis of *pneumocystis jirovecii* and bacterial infection.

The data were analyzed with JMP version 9.0 (SAS institute Japan Ltd, Tokyo, Japan). The Mann–Whitney *U*-test was used for continuous values and the Fisher's exact test for categorical values. Statistical significance was established at $P < 0.05$.

RESULTS

Eleven episodes of agranulocytosis were detected in 11 patients (Table 1). Eight patients developed agranulocytosis after the first rituximab treatment while three others did so only after the second treatment. With the exception of one patient who received four doses of rituximab (Patient 1), all patients

Table 1. Characteristics of patients

Patient	Gender	Age at onset of disease (years)	Age at rituximab treatment (years)	Age at agranulocytosis (years)	Renal biopsy	Previous history of rituximab treatment	Doses of rituximab (375 mg/m^2 for single dose)	Immunosuppressive agents	Sulfamethoxazole/ trimethoprim
1	M	2.5	6.9	7.3	MGA	–	4	CsA	–
2	M	2.3	6.3	6.5	MGA	–	1	MMF, PSL	–
3	M	2.3	4.6	4.8	FSGS	+[a]	1	CsA, PSL	–
4	M	2.0	6.5	6.6	MGA	+[a]	1	CsA, MMF	–
5	F	8.2	11.2	11.3	MGA	–	1	CsA, PSL	–
6	M	6.9	8.1	8.6	MGA	–	1	MMF	–
7	F	3.9	10.2	10.6	MGA	–	1	CsA, MMF	+
8	F	1.3	6.9	7.2	MGA	+[a]	1	CsA, MMF	+
9	M	1.4	3.9	4.3	MGA	–	1	Tac, MZR	+
10	M	2.7	4.4	4.5	MGA	–	1	CsA, MMF	+
11	M	11.6	12.8	13.1	MGA	–	1	MMF	+
Median		2.5	6.9	7.2					

M, male; F, female; MGA, minor glomerular abnormalities; FSGS, focal segmental glomerulosclerosis; CsA, cyclosporin A; Tac, tacrolimus; MMF, mycophenolate mofetil; MZR, mizoribine; PSL, prednisolone.

[a]Patient developed agranulocytosis only after the second treatment of rituximab.

received a single dose. Every patient received one or more immunosuppressive agents at the time of agranulocytosis.

Table 2 shows the clinical features of agranulocytosis in the 11 patients. Median time of onset of agranulocytosis was 66 days (range, 54–161 days) after rituximab treatment. Two patients developed agranulocytosis at the same time as B-cell recovery, while nine patients were accompanied by acute infections and received antibiotics. All except one patient received granulocyte colony-stimulating factor (G-CSF). Agranulocytosis resolved in all cases within a median of 3 days.

The incidence of agranulocytosis was 9.6% (11 of the 114 patients) in total patients and 5.2% (11 of the 213 treatments) in total treatments. Table 3 shows the clinical characteristics of patients with and without agranulocytosis. Median age of the 11 patients who developed agranulocytosis was 6.4 years (interquartile range, 4.3–10.2 years) at the first rituximab treatment; significantly younger than the median age of the 103 patients who did not (median, 12.5 years; interquartile range, 4.3–10.2 years; $P = 0.0009$). Table 4 shows the incidence of agranulocytosis stratified by concomitant immunosuppressive agents. No statistical significance was found among these treatments. Six episodes of agranulocytosis were observed in 129 treatments (4.7%) without the prophylactic use of sulfamethoxazole/trimethoprim, while five episodes were observed in 84 treatments (6.0%) with the use of sulfamethoxazole/trimethoprim (P = not significant).

Five patients (Patient 3, 4, 5, 7 and 9) received re-treatment with rituximab. No recurrence of agranulocytosis was observed in any patient.

DISCUSSION

In this retrospective study, the onset of agranulocytosis was 2–5 months after rituximab treatment and appeared to be associated with infections in most cases. Younger patients were found to be more likely to develop agranulocytosis. Treatment with G-CSF seemed effective. Re-treatment of rituximab was acceptable in all patients.

Table 5 shows previous reports of agranulocytosis associated with rituximab treatment in patients with non-Hodgkin lymphoma [8–17] and autoimmune diseases [18–21]. The incidence of agranulocytosis was reported as 6.9 and 3.2%, respectively. The higher incidence in patients with lymphoma was probably due to the influence of other chemotherapies. In comparison, the incidence in our study is higher than that of patients with autoimmune diseases, but lower than that of patients with lymphoma.

The risk factors for agranulocytosis after rituximab treatment have so far been investigated mainly in lymphoma patients. Autologous stem-cell transplantation [9, 13], acquired immunodeficiency syndrome-related lymphoma [10], previous cytotoxic treatment [11], intensive chemotherapy [13], intensive radiotherapy [13], advanced stage of lymphoma (3 or 4) [13], multiple doses of rituximab (>4) [11] and FcγR genotypes [16] have all been reported to be risk factors. In our study, a young age at time of rituximab treatment seemed to be risk factor. In our study, even though one patient

Table 2. Clinical features of agranulocytosis in 11 patients

Patient	Onset days of agranulocytosis after rituximab treatment	Trigger of finding of agranulocytosis	Ratio of CD19+ B cells in total lymphocytes at agranulocytosis (%)	Nadir neutrophil counts (cells/mm^3)	Maximum C-reactive protein (mg/dL)	Diagnosis of infection	Treatment	Duration of fever (days)	Duration of agranulocytosis (days)	Duration of admission (days)	Days of recovery of B cells after rituximab treatment
1	111	Fever	Unknown[a]	0	7.7	Tonsillitis	PAPM/BP + IVIG + G-CSF	1	3	9	Unknown[c]
2	49	Fever	0.0	10	7.1	FUO	ABPC/SBT + G-CSF	2	4	7	116
3	56	Fever	0.1	54	2.1	URI	CTX + G-CSF	3	6	8	118
4	54	Fever	0.3	27	8.9	FUO	CZOP + G-CSF	2	4	8	115
5	56	By chance[b]	Unknown[a]	72	6.0	FUO	PAPM/BP + G-CSF	2	3	7	117
6	161	Fever	4.6[c]	21	6.9	Sinusitis	CFPM + G-CSF	2	2	9	161
7	140	By chance[b]	3.7[c]	60	7.3	Tonsillitis	CFPM + G-CSF	6	7	14	140
8	104	By chance	0.0	172	< 0.2	No infection	No treatment	–	7	0	Unknown[c]
9	120	Fever	0.1	43	0.7	FUO	CFPM + G-CSF	4	2	9	152
10	63	Fever	0.2	24	4.9	Sinusitis	CFPM + G-CSF	3	4	9	152
11	195	By chance	0.0	336	<0.2	No infection	G-CSF	–	2	11	136
Median	66							2	3	9	136

FUO, fever of unknown origin; URI, upper respiratory infection; IVIG, intravenous immunoglobulin; G-CSF, granulocyte colony-stimulating factor; PAPM/BP, panipenem/betamipron; ABPC/SBT, ampicillin sodium/sulbactam sodium; CTX, cefotaxime sodium; CZOP, cefozopran hydrochloride; CFPM, cefepime dihydrochloride.

[a]Unknown data, patient was followed at other hospitals.

[b]Agranulocytosis was detected by chance in routine blood test at outpatient clinic. Patient developed fever immediately after.

[c]Agranulocytosis developed at the same time as B-cell recovery.

Table 3. Clinical characteristics of patients with and without agranulocytosis

Characteristics	Patients with agranulocytosis ($n = 11$)	Patients without agranulocytosis ($n = 103$)	P-value
Male gender	8 (73%)	68 (66%)	0.75
Age at onset (years)	2.5 (2.1–6.9)	4.8 (2.6–9.3)	0.07
Age at the first RTX treatment (years)	6.4 (4.3–10.2)	12.5 (8.3–15.9)	0.0009
Duration of the disease (years)	3.0 (1.3–4.0)	5.1 (2.5–9.3)	0.01
Renal biopsy (MGA, FSGS, DMP)	10, 1, 0	82, 17, 3a	
Previous therapies			
Prednisolone	11 (100%)	103 (100%)	1
Cyclosporin	11 (100%)	102 (99%)	1
Tacrolimus	1 (9%)	7 (7%)	0.57
Cyclophosphamide	3 (28%)	50 (49%)	0.22
Chlorambucil	0 (0%)	2 (2%)	1
Mizoribine	8 (73%)	89 (86%)	0.21
Mycophenolate mofetil	2 (18%)	14 (14%)	0.65
Azathioprine	0 (0%)	2 (2%)	1
Methylprednisozone pulse therapy	4 (36%)	56 (54%)	0.34
Plasma exchange	1 (9%)	7 (7%)	0.57
Low-density lipoprotein apheresis	0 (0%)	6 (6%)	1

Table 4. Incidence of agranulocytosis stratified by concomitant immunosuppressive agents

	Agranulocytosis (+) $n = 11$	Agranulocytosis (−) $n = 202$	P-value
Cyclosporin			
(+) $n = 113$	9	104	0.06
(−) $n = 100$	2	98	
Tacrolimus			
(+) $n = 6$	1	5	0.28
(−) $n = 207$	10	197	
Mizoribine			
(+) $n = 25$	2	23	0.62
(−) $n = 188$	9	179	
Mycophenolate mofetil			
(+) $n = 149$	7	142	0.73
(−) $n = 64$	4	69	

received four doses of rituximab while the others received a single dose, the number of rituximab may not have been an influencing factor in the development of agranulocytosis. Further investigation is needed to verify this.

While we used G-CSF for our patients, we are aware that its efficacy for rituximab-induced agranulocytosis remains questionable. However, a systematic review of 980 case reports of drug-induced agranulocytosis did demonstrate that the use of hematopoietic growth factors resulted in a shorter median duration of neutropenia, and that patients treated with growth factors had significantly lower proportion of infections or fatal complications [23]. A recent review article proposed that the use G-CSF must be considered on a case-by-case basis [24]. The authors suggested that in patients presenting with Grade 4 neutropenia or associated factors that may confer poor prognosis, such as aged people, absolute neutrophil count of <100 /mm^3, clinical evidence of bacteremia or septic shock and severe comorbidities such as renal failure, the use of G-CSF may be justifiable.

To date, there are few reports on re-administration of rituximab. One case series from Israel reported a rituximab re-treatment for late-onset neutropenia resulted in recurrent episodes of agranulocytosis in one patient [25], while another case series reported six patients with re-treatment but had no such recurrence [21]. In our study, none of the patients experienced recurrence of agranulocytosis. Moreover, 3 of the 11 patients developed agranulocytosis only after the second rituximab treatment. Nonetheless, further investigation is required to confirm the safety of rituximab re-treatment for patients with a history of rituximab-associated agranulocytosis.

The exact mechanism of neutropenia associated with rituximab remains poorly understood. Several hypotheses have been proposed, such as the transient production of autoantibodies against neutrophils during immune reconstitution [26], hyperproliferation of large granular T cells in the bone marrow [27, 28] or viral infection during dysfunctional humoral immunity [29, 30]. In previous reports, neutrophil maturation arrest was revealed by bone marrow aspiration. A direct toxicity of rituximab is less likely since CD20 is not expressed on neutrophils and their precursors. Dunleavy *et al.* [10] reported a correlation between rapid B-cell recovery and perturbation of stromal cell-derived factor-1 (SDF-1) and neutropenia. Another cytokine influenced by rituximab administration is B-cell-activating factor (BAFF), which plays a role in human B-cell survival, expansion and development. Terrier *et al.* [31] reported that BAFF levels were undetectable prior to rituximab administration, but increased after treatment and eventually peaked at a time interval that coincided with the occurrence of neutropenia. The fact that two of our patients developed agranulocytosis almost at the same time as B-cell recovery may support these hypotheses, although it is noteworthy that neither SDF-1 nor BAFF was analyzed. Some reports have shown that FcγRIIIa polymorphism was highly associated with the development of late-onset neutropenia [16, 32]. Weissmann-Brenner *et al.* [33], who showed a suppression of colony-forming unit growth by plasma of patients with rituximab-induced neutropenia, have proved that circulating antibodies in the plasma may be responsible for leukopenia after rituximab treatment. Again, further investigation is necessary to confirm the exact pathogenesis.

Agranulocytosis was detected in four of our patients by chance, supporting the theory of a primary mechanism. The other seven patients experienced fever prior to detection, suggesting a mechanism secondary to infections. On the other hand, 32 events of bacterial infections were observed in the 114 patients who received rituximab treatments but experienced no agranulocytosis. As such, we think that the cause of agranulocytosis varies from patient to patient.

There are several limitations to our study. First, we do not have information on the maturation of neutrophils as no bone marrow aspirations were performed. Second, as the patients were receiving one or more immunosuppressive agents at the

Table 5. Previous reports of agranulocytosis associated with rituximab treatment

Reference	Years of publication	Primary disease	Number of patients of rituximab treatment	Number of patients of agranulocytosis	Incidence of agranulocytosis (%)
[8]	2003	NHL	59	8	14
[9]	2004	NHL	39	6	15
[10]	2005	NHL	76	6	8
[11]	2006	NHL	77	10	13
[12]	2006	NHL	54	3	6
[13]	2006	NHL	107	10	9
[14]	2008	NHL	113	6	5
[15]	2009	NHL	121	4	3
[16]	2010	NHL	80	2	3
[17]	2012	NHL	160	6	4
[18]	2007	AID	23	1	4
[19]	2009	AID	65	2	3
[20]	2011	AID	209	5	2
[21]	2012	AID	138	6	4

time of agranulocytosis, we cannot rule out the possibility that these agents may have an association with the condition. However, none of our patients developed agranulocytosis before rituximab use despite the use of a similar immunosuppressive menu. This suggests that rituximab is likely to be the main cause of agranulocytosis. Third, the number of patients was relatively small. We need to accumulate more clinical data and information on this complication in future studies.

In conclusion, rituximab use is a potential cause of agranulocytosis for patients with refractory idiopathic nephrotic syndrome. As rituximab use is increasing in such patients, we have to be vigilant of any potential clinically serious delayed-onset complication. When a patient with a history of rituximab treatment suffers from fever, we should not rule out the possibility of agranulocytosis as well as serious bacterial infections such as sepsis. At the same time, it is necessary for patients to recognize this complication and visit an emergency clinic as soon as possible upon developing fever. The monitoring of complete blood counts and CD19+ B-cell counts at least once a month after rituximab use until B-cell recovery may be helpful for the early detection of agranulocytosis and prevention of serious infection. Further clinical studies are needed to elucidate the pathogenesis of delayed-onset postrituximab agranulocytosis.

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CONFLICT OF INTEREST STATEMENT

None declared.

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