A randomized clinical trial indicates that levamisole increases the time to relapse in children with steroid-sensitive idiopathic nephrotic syndrome

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Mariken P. Gruppen[1], Antonia H. Bouts[1], Marijke C. Jansen-van der Weide[2], Maruschka P. Merkus[3], Aleksandra Zurowska[4], Michal Maternik[4], Laura Massella[5], Francesco Emma[5], Patrick Niaudet[6], Elisabeth A.M. Cornelissen[7], Thierry Schurmans[8], Ann Raes[9], Johan van de Walle[9], Mieke van Dyck[10], Ashima Gulati[11], Arvind Bagga[11] and Jean-Claude Davin[1,8]; all members of the Levamisole Study Group

[1]Department of Pediatric Nephrology, Emma Children's Hospital/Academic Medical Center, Amsterdam, The Netherlands; [2]Pediatric Clinical Research Office, Children's Hospital/Academic Medical Center, Amsterdam, The Netherlands; [3]Clinical Research Unit, Academic Medical Center, Amsterdam, The Netherlands; [4]Department of Pediatric and Adolescent Nephrology and Hypertension, Medical University of Gdansk, Gdansk, Poland; [5]Nephrology and Dialysis Unit, Pediatric Subspecialties Department, Bambino Gesù Children's Hospital, IRCCS, Rome, Italy; [6]Service de Néphrologie Pédiatrique, Hôpital Necker-Enfants Malades, Paris, France; [7]Department of Pediatric Nephrology, Radboud University Medical Centre, Amalia Children's Hospital, Nijmegen, The Netherlands; [8]Department of Pediatric Nephrology, Hôpital Universitaire des Enfants Reine Fabiola, Brussels, Belgium; [9]Department Pediatric Nephrology, Ghent University Hospital, Ghent, Belgium; [10]Department of Pediatrics, University Hospital, Leuven, Belgium; and [11]Division of Nephrology, Department of Pediatrics, All India Institute of Medical Sciences, New Delhi, India

Levamisole has been considered the least toxic and least expensive steroid-sparing drug for preventing relapses of steroid-sensitive idiopathic nephrotic syndrome (SSINS). However, evidence for this is limited as previous randomized clinical trials were found to have methodological limitations. Therefore, we conducted an international multicenter, placebo-controlled, double-blind, randomized clinical trial to reassess its usefulness in prevention of relapses in children with SSINS. The efficacy and safety of one year of levamisole treatment in children with SSINS and frequent relapses were evaluated. The primary analysis cohort consisted of 99 patients from 6 countries. Between 100 days and 12 months after the start of study medication, the time to relapse (primary endpoint) was significantly increased in the levamisole compared to the placebo group (hazard ratio 0.22 [95% confidence interval 0.11–0.43]). Significantly, after 12 months of treatment, six percent of placebo patients versus 26 percent of levamisole patients were still in remission. During this period, the most frequent serious adverse event (four of 50 patients) possibly related to levamisole was asymptomatic moderate neutropenia, which was reversible spontaneously or after treatment discontinuation. Thus, in children with SSINS and frequent relapses, levamisole prolonged the time to relapse and also prevented recurrence during one year of treatment compared to prednisone alone. However, regular blood controls are necessary for safety issues.

Idiopathic nephrotic syndrome (INS) is a rare disease with an incidence that varies between 2 and 7 cases per 100,000 children per year.[1,2] It is hypothesized that INS results from a defect in lymphocyte function.[3] Most children with INS are steroid sensitive. Seventy percent of the latter also experience ≥1 relapses.[1,2] Half of these children relapse frequently after cessation of corticosteroids (frequently relapsing nephrotic syndrome [FRNS]) or become steroid dependent.[4] Children with FRNS are then exposed to the side effects of steroids.[1,2] To reduce the relapse rate, several drugs have been used.[1,2] Among these, levamisole was considered the least toxic and least expensive and the only one not classified as an immunosuppressive agent until the pharmaceutical industry withdrew it from the market in 2004 for human use due to lack of clear indications.[5] Evidence of the efficacy of levamisole in FRNS was restricted to retrospective studies and a few clinical trials, all of which had some methodological limitations.[6–8] Since then, it has only remained available as a low-cost drug for veterinary use, given of its anthelminthic properties. We hypothesized that the addition of levamisole following complete remission with steroid therapy in children with FRNS or steroid dependency

Correspondence: Mariken P. Gruppen, Emma Children's Hospital, Pediatric Nephrology, Academic Medical Center, Meibergdreef 15, Amsterdam, North Holland 1100 DD, The Netherlands. E-mail: m.p.gruppen@amc.nl

would either prevent relapses or prolong the time to relapse during a 1-year treatment period.

For this reason, we conducted an international, multicenter, double-blind, placebo-controlled, randomized clinical trial (RCT) to assess the efficacy and safety of 1-year levamisole treatment in children with FRNS. Study medication (levamisole or placebo) started during prednisone treatment for a relapse. To study longer term efficacy and safety of levamisole, patients still in remission and on levamisole treatment at trial completion were evaluated in an additional longer term follow-up.

RESULTS
Patients
Between October 2007 and March 2012, 103 patients, recruited from 13 sites in 6 countries (The Netherlands, Belgium, France, Italy, Poland, and India), were randomized. Ninety-nine patients were included in the modified intention-to-treat population. Three randomized patients could not be included because they did not start the study medication; for 2 of them, the study medication (placebo) did not arrive on time, and proteinuria developed in the third patient (levamisole) before the start of study medication. One patient (placebo) was excluded due to missing primary outcome information. Eight patients prematurely discontinued study medication (4 levamisole and 4 placebo) and were censored. No patients were lost to follow-up (Figure 1). Nonadherence with study treatment was not detected in any patient.

Demographic and baseline characteristics were similar across both study treatment groups (Table 1). Analysis of age and steroid dependency (SD) by region showed a similar age distribution for both regions, but a difference in the distribution of SD between Europe and India (Table 2). The range and distribution of the lowest prednisone doses necessary to prevent a relapse before the start of study are depicted in the Supplementary Material Table S1.

Efficacy endpoints
Time to relapse (primary endpoint). Kaplan-Meier analysis in the primary analysis (modified intention-to-treat) population showed similar cumulative relapse-free survival probabilities for both treatment groups during the first 100 days of study medication (Figure 2). However, afterward, a difference in the time to relapse became apparent in both treatment groups in favor of the levamisole group (log-rank analysis, total period, $P = 0.015$). By the end of the 1-year study period, 6% of placebo (3/49) versus 26% (13/50) of levamisole patients were still in remission and on study medication (Figure 2). Since Kaplan-Meier curves showed that proportional hazards could not be assumed throughout the 1-year study period, a time-dependent Cox proportional hazards regression analysis was performed incorporating the stratification factors. During the first 100 days of study medication similar hazards for a relapse needing prednisone were observed in both treatment groups (hazard ratio [HR]: 1.14, 95% confidence interval [CI] 0.56–2.34, $P = 0.72$).
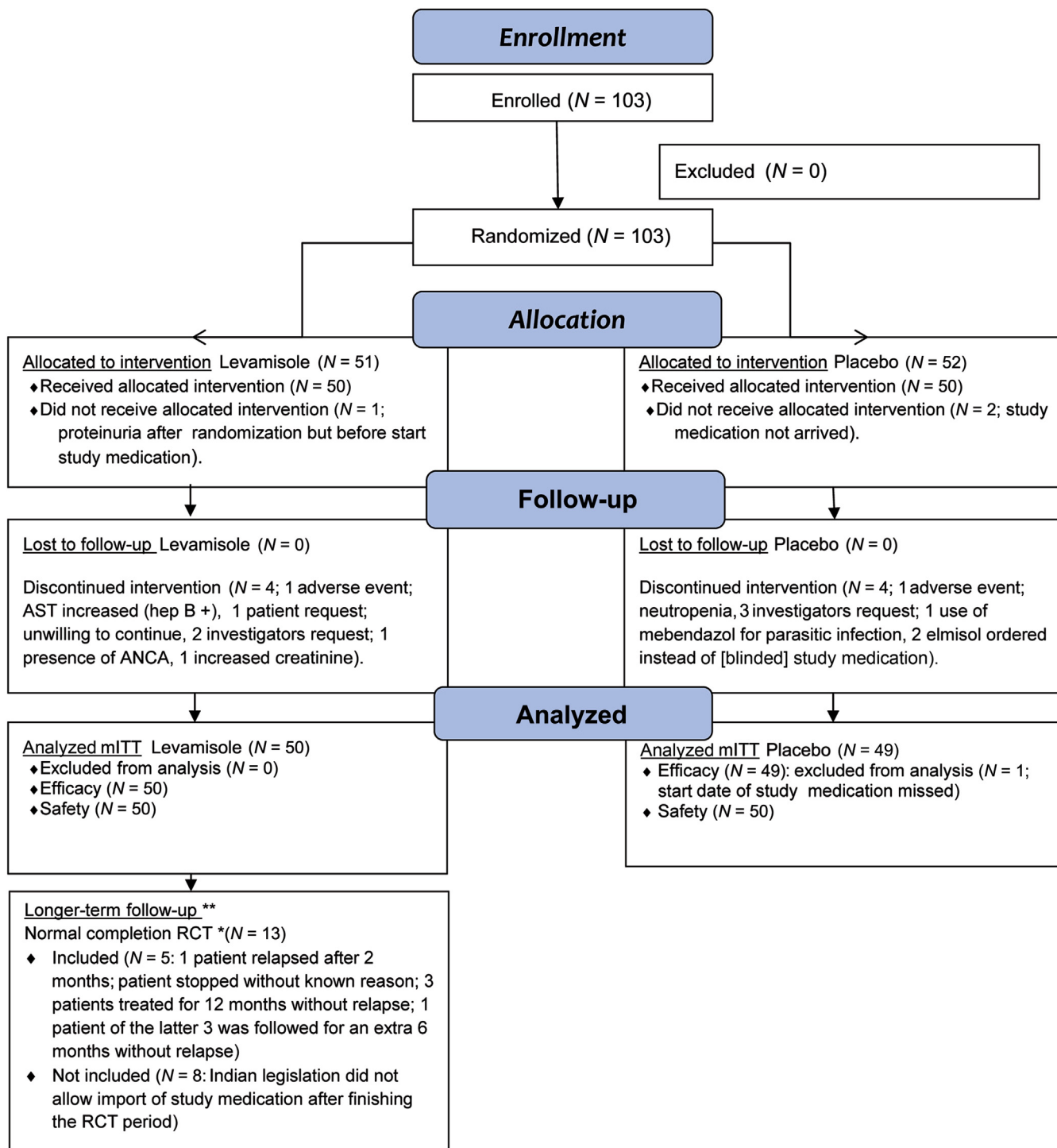
Afterward, a significantly lower hazard was observed in the levamisole group compared with the placebo group (HR: 0.22, 95% CI 0.11–0.43, $P = 0.001$) (Table 3). The HR was not confounded by the prespecified covariates of sex, age, and ethnicity. Exploratory subgroup analyses suggested insufficient evidence of treatment effect modification of the time to relapse by region (India vs. Europe), SD (yes vs. no), sex, and age (2–5 years vs. ≥6 years) (formal tests for treatment by subgroup interaction; P values of 0.79, 0,64, 0.35, and 0.51, respectively); see Kaplan-Meier curves for region and SD/FR in the Supplementary Material Figures S1 and S2.

Occurrence of prednisone-needing relapse (secondary endpoint). At 1-year follow-up, a lower cumulative proportion of patients with a relapse requiring prednisone was seen in the levamisole group (33/50, 66%) compared with the placebo group (42/49, 86%) (relative risk estimate/crude relative risk, 0.77; 95% CI 0.61–0.97; after adjustment for the stratification variables, adjusted odds ratio was 0.30; 95% CI 0.11–0.82, $P = 0.02$). Formal evaluation of effect modification using treatment by subgroup interaction terms in multivariable regression models, including the stratification variables, suggested insufficient evidence of heterogeneity of treatment effect between these subgroups (Supplementary Material Table S2).

Safety endpoints
In the safety population, more patients with at least 1 adverse event (AE) were seen in the levamisole group versus the placebo group (levamisole, 58% [29/50] vs. placebo, 38% [19/50], $p = 0.045$). However, the most clinically prominent AEs were similar in both groups (pyrexia, nasopharyngitis, cough, and mild neutropenia; 1000–1500 cells/μl in 3 patients of each group). None of the latter required study discontinuation (Table 4). Ten nonlethal serious AE occurred. They included 5 cases of moderate neutropenia (500–1000 cells/μl) (levamisole, 4/50 vs. placebo, 1/50) that were asymptomatic and reversible after levamisole discontinuation (2/4 levamisole group) or spontaneously (2/4 levamisole group); 3 hospitalizations (levamisole, 3/50 vs. placebo, 0/50), not related with neutropenia; 2 levamisole patients presented with reversible side effects after medication discontinuation, 1 with a reduced glomerular filtration rate and 1 with arthritis and antineutrophil cytoplasmic antibodies. With regard to the 4 levamisole patients with moderate neutropenia, in 2 of them, neutropenia resolved without terminating levamisole and both reached normal completion of the trial. The other 2 levamisole patients with neutropenia presented with a relapse at the same time, so their primary endpoint was reached and study medication was terminated. With respect to the 3 levamisole patients who required hospitalization (for high fever in 1, pulmonary infection in 1, and abdominal pain in 1), 2 presented with relapse at the same time, so trial medication was discontinued, whereas the third patient did not end trial medication but relapsed 8 months later (Table 4).

Comparison of laboratory values per visit (hemoglobin, platelets, neutrophils, albumin, aspartate aminotransferase,

Enrollment

Enrolled (*N* = 103)

Excluded (*N* = 0)

Randomized (*N* = 103)

Allocation

Allocated to intervention Levamisole (*N* = 51)
♦ Received allocated intervention (*N* = 50)
♦ Did not receive allocated intervention (*N* = 1; proteinuria after randomization but before start study medication).

Allocated to intervention Placebo (*N* = 52)
♦ Received allocated intervention (*N* = 50)
♦ Did not receive allocated intervention (*N* = 2; study medication not arrived).

Follow-up

Lost to follow-up Levamisole (*N* = 0)

Discontinued intervention (*N* = 4; 1 adverse event; AST increased (hep B +), 1 patient request; unwilling to continue, 2 investigators request; 1 presence of ANCA, 1 increased creatinine).

Lost to follow-up Placebo (*N* = 0)

Discontinued intervention (*N* = 4; 1 adverse event; neutropenia, 3 investigators request; 1 use of mebendazol for parasitic infection, 2 elmisol ordered instead of [blinded] study medication).

Analyzed

Analyzed mITT Levamisole (*N* = 50)
♦ Excluded from analysis (*N* = 0)
♦ Efficacy (*N* = 50)
♦ Safety (*N* = 50)

Analyzed mITT Placebo (*N* = 49)
♦ Efficacy (*N* = 49): excluded from analysis (*N* = 1; start date of study medication missed)
♦ Safety (*N* = 50)

Longer-term follow-up **
Normal completion RCT *(*N* = 13)
♦ Included (*N* = 5: 1 patient relapsed after 2 months; patient stopped without known reason; 3 patients treated for 12 months without relapse; 1 patient of the latter 3 was followed for an extra 6 months without relapse)
♦ Not included (*N* = 8: Indian legislation did not allow import of study medication after finishing the RCT period)

Figure 1 | Randomization and follow-up of the study participants. *Normal completion: patients who were still in remission and on study medication at 12 months after start study medication. **Longer term follow-up: to evaluate whether the effect of levamisole remained constant or diminished over time and to assess side effects of long-term levamisole treatment; patients in remission at trial completion and still receiving levamisole were followed for another 18 months (12 months with and subsequently 6 months without levamisole). ANCA, antineutrophil cytoplasmic antibody; AST, aspartate aminotransferase; hep, hepatitis; mITT, modified intention-to-treat; RCT, randomized clinical trial.

and glomerular filtration rate), categorized as high, low, or normal according to age, did not show any significant difference between the 2 treatment groups except at visit 3. At that time, a significantly higher number of low glomerular filtration rate values could be observed for the levamisole group ($P = 0.03$). The normal and abnormal values for the glomerular filtration rate, neutrophils, and aspartate aminotransferase according to time are shown in the Supplementary

Table 1 | Baseline characteristics of the modified intention-to-treat population

Baseline characteristics	Placebo (N = 49)	Levamisole (N = 50)
Cyclophosphamide, N (%)[a]	3 (6)	3 (6)
Steroid-dependence, N (%)	32 (65)	36 (72)
Lowest prednisone, mean (SD)[b]	21.16 (12.73) mg/m²/AD	26.33 (16.42) mg/m²/AD
Region, N (%)		
France	4 (8)	5 (10)
Rest of Europe	24 (49)	23 (46)
India	21 (43)	22 (44)
Sex, boys, N (%)	34 (69)	36 (72)
Ethnicity, N (%)		
Caucasian	25 (51)	25 (50)
Black	2 (4)	0 (0)
Asian	21 (43)	23 (46)
Other	1 (2)	1 (2)
Missing	0 (0)	1 (2)
Age at presentation of NS (yr), median (IQR)	3.5 (2.25–5.75)	4 (2–5.25)
Age at randomization (yr), mean (SD)	6.0 (3.1)	5.7 (2.6)
Duration disease (yr),[c] median (IQR)	1 (0–2)	1 (0–2)

AD, alternate day; IQR, interquartile range; NS, nephrotic syndrome.
[a]Previous cyclophosphamide treatment.
[b]Lowest mean prednisone dose necessary to prevent a relapse before inclusion in the randomized clinical trial.
[c]Age at presentation of newly diagnosed nephrotic syndrome.

Figure 2 | Relapse-free survival (Kaplan-Meier curve). The cumulative proportion of patients in the modified intention-to-treat population remaining in remission and on study medication over time from the start of trial medication through a 1-year follow-up period of the randomized clinical trial. The vertical bars on both curves indicate study discontinuation for reasons other than relapse (i.e., censored patients). L, levamisole; P, placebo.

Material Figures S3–S5. No difference in delta height between baseline and 1-year follow-up was observed between the levamisole and placebo groups (Supplementary Material Figure S6).
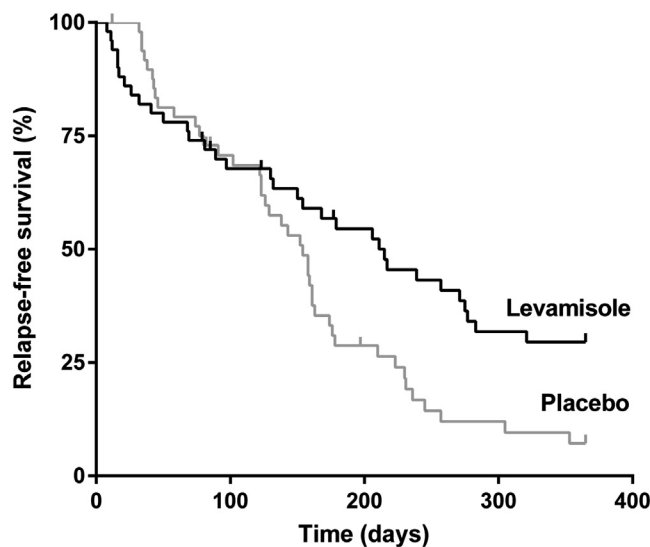
Longer-term follow-up

Of the 13 levamisole patients who did not present with a relapse during the year of the RCT, only 5 could subsequently be included in the longer term follow-up extension of the trial (Figure 1). Regarding the other 8 patients, all Indian, Indian legislation did not allow import of study medication after completion of the trial period because local Indian companies have been producing levamisole for almost 3 decades. Of the

Table 2 | Age and steroid dependence distribution in the European and Indian subgroups of the modified intention-to-treat population

Age/steroid dependence	European countries (N = 56)	India (N = 43)
Age, yr,[a]		
2–5	24 (43%)	23 (54%)
6–16	32 (57%)	20 (47%)
Steroid dependence[b]		
Yes	50 (89%)	18 (42%)
No	6 (11%)	25 (58%)

[a]χ² test, P = 0.29.
[b]χ² test, P < 0.0001.

5 included patients, 1 had a relapse after 2 months and 1 stopped study participation after only 4 months (reason unknown). The 3 remaining patients were treated with levamisole for 12 months in the longer term follow-up without a relapse; of those, only 1 was followed for 6 months after levamisole cessation and did not relapse. One serious AE was reported: asymptomatic neutropenia, <500 cells/µl, which resolved after levamisole termination.

DISCUSSION

This study shows that the addition of levamisole to standard steroid therapy in children with FRNS increased the time to relapse, after termination of prednisone treatment (100 days post-randomization). Furthermore, it allowed 26% of levamisole patients to be free of relapse for at least 1 year (vs. 6% in the placebo group). These results confirm the efficacy of levamisole in FRNS, suggested previously by several retrospective studies and 3 RCTs that had methodological limitations to some extent.[6–8] Two RCTs[7,8] had unclear allocation concealment, no blinding of patients and investigators, insufficient statistical power, and inadequate relapse definitions (i.e., not defined as a relapse necessitating prednisone treatment). The British Association of Pediatric Nephrology (BAPN) trial[6] had adequate allocation concealment and was double-blinded and placebo-controlled; however, levamisole was given for only 4 months. In this study, more than half of

Table 3 | Time to relapse (primary outcome) in the mITT population: results of the time-dependent Cox proportional hazards regression model

	Levamisole (N = 50)	Placebo (N = 49)	Levamisole vs. placebo, hazard ratio (95% CI)[a]	P value
No. of patients with a relapse needing prednisone				
<100 days of follow-up	16	14	Crude: 1.19 (0.58–2.43)	0.64
			Adjusted[b]: 1.14 (0.56–2.34)	0.72
≥100 days of follow-up	17	28	Crude: 0.34 (0.18–0.63)	0.001
			Adjusted[b]: 0.22 (0.11–0.43)	<0.001

CI, confidence interval; mITT, modified intention-to-treat.
[a]Hazard ratios were calculated using a time-dependent Cox proportional hazards regression model with corresponding 95% confidence intervals. The hazard ratio can be interpreted as the relative risk of the outcome at any fixed point in time.
[b]Adjusted for the stratification factors.

the patients had received cyclophosphamide, which might have biased the evaluation of levamisole effect because it has been suggested that levamisole may be more effective in patients previously treated with alkylating therapy.[9] In the latter trial, a difference in efficacy between the levamisole and the placebo groups became apparent after 40 days, in contrast to our trial in which a beneficial effect appeared after 100 days. The difference in efficacy between the BAPN and our trial could be the result of a greater dosage and longer steroid treatment in our study (cumulative prednisone dosage, alternate days: BAPN trial, 24.5 mg/kg during 10 weeks vs. levamisole trial, 2000 mg/m^2 during 16 weeks). This is illustrated by a higher percentage of control patients in remission at 100 days in our study compared with the BAPN study (percentage of control subjects in remission on day 100 in the levamisole trial was 70% vs. BAPN, <20%).

Table 4 | Most frequently encountered SAEs in the safety population

	Levamisole (n/N)	Placebo (n/N)
AEs		
At least 1 AE[a]	29/50	19/50
Cough	6/50	6/50
Nasopharyngitis	8/50	10/50
Pyrexia	10/50	6/50
Neutropenia (1000–1500/μl)	3/50	3/50
SAEs		
Neutropenia (500–1000/μl)	4/50	1/50
Neutropenia (<500/μl)	1/50	
Hospitalization[b]	3/50[a]	0/50
Reduced GFR	1/50	0/50
Arthritis/ANCA+	1/50	0/50

AEs, adverse events; ANCA, antineutrophil cytoplasmic antibody; GFR, glomerular filtration rate; SAEs, serious adverse events.
[a]Pulmonary infection, fever, abdominal pain.
[b]P = 0.045

The currently observed increased efficacy of levamisole after day 100 that coincided with the cessation of prednisone might result from an increased metabolism and decreased bioavailability of levamisole due to steroids as has been shown for cyclosporine, tacrolimus, and sirolimus.[10] Although levamisole is not classified as an immunosuppressive agent, investigation of possible metabolic and transporter pathways with prednisone and duration of prednisone treatment, regarding concentration levels, has not yet been investigated.

Exploratory descriptive analyses in separate subgroups suggested possible differential treatment effects for subgroups of the region and steroid dependency (Supplementary Material Figures S1 and S2 and Table S2). However, formal evaluation of effect modification using treatment-by-subgroup interaction terms in multivariable regression models, including stratification variables, suggested insufficient evidence of heterogeneity of treatment effect between these subgroups. Looking at the distribution of SD patients in the European subgroup compared with the Indian subgroup, the greater majority of the European group (89%) involved SD patients, whereas in the Indian subgroup, a proportion of 42% involved SD patients. This may have resulted in confounding, which is obscured in the descriptive within-subgroups analyses. Moreover, these subgroup analyses should be interpreted with much caution as these are hampered by a lack of statistical power to adequately analyze differential treatment effects between subgroups. This RCT was only sufficiently powered to assess the overall treatment effect. Additionally, the issue of multiplicity of testing, which increases the probability of false-positive findings, related to subgroup analyses should be acknowledged. Therefore, the results of the exploratory subgroup analyses should only be considered as a hypothesis-generating exercise, needing establishment by future adequately powered RCTs.

So far, earlier RCTs on levamisole only included SD patients[6] or did not report the results of SD and frequently relapsing patients separately.[7,8]

The smaller proportion of included SD patients in India compared with Europe is consistent with a recent retrospective study from this country.[11] It might be explained by genetic predisposition and/or environmental factors.[12,13] The small proportion of frequently relapsing patients in the European group may be explained by a more frequent use of the French prednisone protocol for a relapse in Europe. In this protocol, relapse treatment includes 4 months of steroids on alternate days in addition to the period of daily treatment until remission. Following this protocol, it is practically impossible to fit with the definition of frequently relapsing that requires at least 4 relapses per year, even if the protocol is adapted individually.

In the safety population, asymptomatic and reversible neutropenia was reported in 4 placebo patients (4/50, 8%) and in 7 levamisole patients (7/50, 14%) (3 mild and 4 moderate cases) during the RCT year as well as in 1 patient (severe) in the longer term follow-up phase. All patients were asymptomatic and reversible after discontinuation of the drug

or spontaneously in 2 patients. Although complete blood counts were obtained every 2 to 4 weeks[6,7] or 3 times a month,[8] neutropenia was not reported in 3 other RCTs.[6–8] In 7 cohort studies (2 RCTs and 5 cohort studies), largely from Middle East countries and India, neutropenia was only reported in 8 of a total of 573 included patients in these studies.[14–20] Most of these studies checked the blood counts on a regular basis (biweekly or monthly), but Dayal *et al.*[8] and Ekambaram *et al.*[15] analyzed blood counts every 3 months. Madani *et al.*,[17] who included the majority of the patients (304/573 patients), did not report the frequency of follow-up visits. Because neutropenia during levamisole treatment could be reversible and present without clinical symptoms, neutropenia could have been missed in these studies.[8,15,17]

The strengths of our trial include the international, multicenter, double-blind, placebo-controlled comparison of 1-year levamisole treatment in a large sample of a relatively rare disease population. The compliance with study medication was regularly controlled, and nonadherence was not detected in any patient. However, some limitations of our trial must be noted. A few patients discontinued study medication prematurely for other reasons than a relapse and were censored. However, because this concerned only a few and equal number of patients in both study groups (4 in each group), we believe that the eventual influence of informative censoring on the estimated treatment effects can be assumed to be minimal. Because only 5 levamisole patients could be included in the longer term follow-up phase, this study lacks information on the long-term efficacy and safety of levamisole. Unfortunately, information on the cumulative steroid dose and the number of patients who were evaluated for eligibility but did not meet the eligibility criteria or who met the eligibility criteria but declined to be enrolled is lacking. It should be noted that this investigator-initiated, international pediatric trial lacked financial resources to fund designated local research staff to assist the investigators, who conducted this study on a voluntary basis in addition to their busy day-to-day clinical practice.

Patients with FRNS often need to be treated for many years with different steroid-sparing agents. It is reasonable to start with the drug with the best efficacy versus safety profile. Because of a high rate of diverse serious side effects, it is obvious that calcineurin inhibitor agents should be reserved only in case of the failure of other drugs. Cyclophosphamide was the first agent to be successfully used as a steroid-sparing agent. A meta-analysis of RCTs comparing levamisole and cyclophosphamide did not show a clear difference in efficacy and side effects except for a prolonged treatment effect after drug discontinuation of cyclophosphamide but not levamisole.[21] However, a meta-analysis concludes that cyclophosphamide use in steroid-sparing INS is associated with a higher risk of leucopenia (1 in 3 risk) and that oligospermia cannot be excluded for any dose of cyclophosphamide.[22] To the best of our knowledge, until now, there was no study comparing levamisole with mycophenolate. Levamisole is not an immunosuppressive drug but an immunomodulator

and immunostimulant. It is particularly important for children needing prolonged courses of different steroid-sparing agents in order to reduce the side effects of cumulative immunosuppression.

To summarize, addition of levamisole to steroid therapy led to prolongation of time to relapse in children with FRNS from 5 European countries and India. Neutropenia was the most frequently observed adverse effect but was always asymptomatic and reversible spontaneously or with levamisole discontinuation. Other very rare, severe levamisole side effects previously reported in the treatment of INS, including hepatitis, convulsions, and antineutrophil cytoplasmic antibody vasculitis, were not observed in this study. However, an antineutrophil cytoplasmic antibody–associated arthritis reversible with levamisole discontinuation was observed in 1 patient. An unexpected and unexplained reduction of the creatinine clearance that was immediately and completely reversible after discontinuation of the drug was also observed in 1 patient.

Because regular blood controls allowed early detection and reversibility without complications of all levamisole-related side effects, this trial justifies the use of levamisole as the first second-line drug in children with FRNS to prevent relapses and lessen the complications of steroids.

Future adequately powered trials should be performed to evaluate the efficacy and safety of levamisole in subgroups of FRNS (i.e., without and with different degrees of steroid dependence).

METHODS
Trial design and oversight
We performed an international, multicenter, placebo-controlled, double-blind, randomized clinical superiority trial, followed by a longer term follow-up study to evaluate the efficacy and safety of levamisole in children with INS.

Ethical approval of the study protocol was obtained from the Institutional Review Boards of each participating institute. The study was prospectively registered in international trial registers (Eudra-CT/EMA register 9/2007, Eudra CT 2005-005745-18, NTR1769, ISRCTN 23853712).

Details on design and oversight are provided in the Supplementary Material (item 2).

Patients and participating centers
FRNS children with or without steroid dependence (SD, FR), 2 to 18 years of age were eligible for the study. FRNS was defined as ≥2 relapses occurring within 6 months of the initial response or ≥4 relapses occurring in any 12-month period; SD was based on the report of any lowest prednisone dose needed to prevent a relapse before inclusion in the trial (Table 5).

Exclusion criteria included NS with kidney disease, neutropenia, convulsions, hepatic disease, unresponsiveness to cyclosporine or mycophenolate mofetil, prolongation of the Qtc interval on surface electrocardiography (>0.44 s) at presentation, pregnancy, breastfeeding, participation in another trial as well as those patients previously treated with levamisole. Patients were recruited from 13 academic hospitals in 6 countries, including The

Table 5 | Definitions

Condition	Diagnostic criteria
Adverse event	Any adverse change in condition between the time of informed consent and end of the trial
Idiopathic nephrotic syndrome	Nephrotic range proteinuria (urinary proteins >200 mg/mmol creatinine) with hypoalbuminemia (serum albumin <25 g/l) without signs of a specific etiology (e.g., Henoch-Schoenlein purpura, acute post-infectious glomerulonephritis)
Frequent relapses	≥2 relapses occur within 6 months of initial response or when ≥4 relapses occur in any 12-month period
Neutropenia	According to the protocol, we stopped the study medication for an absolute neutrophil count <1500/μl; a neutrophil count of <1000 cells/μl was considered an SAE. Mild neutropenia: >1000 and <1500 cells/μl. Moderate neutropenia: >500 and <1000 cells/μl. Severe neutropenia: <500 cells/μl.
Prolonged Qtc	Heart rate–corrected QT interval (QTc) >0.44 seconds, calculated with Bazett's formula
Remission	Proteinuria is <20 mg/mmol creatinine or at most a trace of protein when tested by dipstick on at least 3 consecutive days
Serious adverse event	Any untoward medical occurrence or effect that at any dose: - results in death - is life threatening - requires hospitalization or prolongation of existing inpatients' hospitalization - results in persistent or significant disability or incapacity - is a congenital anomaly or birth defect Any other important medical event that may not result in death or be life threatening or require hospitalization may be considered a serious adverse experience when, based on appropriate medical judgment, the event may jeopardize the subject or may require an intervention to prevent one of the outcomes listed.
Steroid dependence	Relapse occurs during prednisone reduction or within 2 weeks after corticosteroid cessation. Steroid dependency is based on any reported lowest prednisone dose needed to prevent a relapse before inclusion in the trial.
Steroid-sensitive nephrotic syndrome	Treatment of idiopathic syndrome with corticosteroids that leads to remission.

Netherlands, Belgium, France, Italy, Poland, and India. Before enrollment, written informed consent to participate in the study was obtained from all patients and/or their parent(s)/legally acceptable representative.

Randomization and blinding

Patients were randomly allocated, when their urine was protein free for 1 day and after signing the informed consent, to 1 of the 2 study medications by a computerized random number generator in a 1:1 ratio. The randomization was stratified by SD (yes/no), previous use of cyclophosphamide (yes/no), and region (France, other European countries, India). Within the strata, the randomization procedure was blocked. Patients, parents, investigators, medical and nursing staff, outcome assessors, monitors, and data analysts were blinded to study medication. Randomization data were kept strictly confidential and accessible only to authorized persons.

Study intervention and procedures

Prednisone was started at the relapse of nephrotic syndrome, according to dosage and tapering in steroid-sparing INS treatment protocol of the French Society of Pediatric Nephrology (Table 6). This protocol was chosen, due to the absence of an internationally accepted guideline. Our choice was based on a meta-analysis suggesting that longer steroid treatments and higher cumulative dosage would be expected to better reduce the risk of relapse in steroid-sparing INS.[23] Levamisole or a matched placebo was started at remission after at least 3 days, but not later than 21 days. The long interval was necessary to distribute the study medication to India.

Levamisole was given orally as a tablet at 2.5 mg/kg on alternate days, with a maximum dose of 150 mg. In order to obtain accurate dosing in all children, 4 tablet strengths had been formulated (i.e., tablets containing 5, 10, 25, and 50 mg of levamisole.[24] Proteinuria was checked once a week by the parents by means of dipstick and also in case of infection or clinical edema.

Individual unblinding took place when a relapse occurred (to give patients in the placebo group the possibility of receiving levamisole) or at the end of the 12-month trial.

Treatment was discontinued when the primary outcome was reached, in case of a serious AE, or at the patient's request. When a relapse occurred before 1 year of follow-up, clinical follow-up was done every 3 months for 1 year from the start of study medication.

Individual patient trial medication compliance was checked by the investigator every 3 months, along with the number of dispensed and returned tablets, using a drug accountability form.

More details on the study intervention and visits are given in the Supplementary Material (item 3, Tables S3 and S4).

Outcome measures

Time to relapse (primary endpoint). The primary endpoint was the time to relapse (i.e., the time between the start of study medication and the occurrence of a relapse). Patients who did not experience a relapse during the 1-year follow-up period of the RCT were censored at 1 year after the start of study medication. Only a relapse necessitating prednisone treatment was considered a primary endpoint relapse.

Table 6 | Prednisone treatment of steroid-sensitive nephrotic syndrome relapse

Dosage	Period of use
60 mg/m² once daily	Once daily until urine is protein free for 6–8 days
60 mg/m² once daily	Every other day for 4 weeks
45 mg/m² once daily	Every other day for 4 weeks
30 mg/m² once daily	Every other day for 4 weeks
15 mg/m² once daily	Every other day for 4 weeks

Corticosteroids at inclusion are given according to the national French protocol for treatment of a relapse of steroid-sensitive nephrotic syndrome (www.soc-nephrologie.org).

Occurrence of a prednisone-needing relapses (secondary endpoint).   The key secondary outcome concerned the cumulative number of study discontinuations for a relapse needing prednisone during the 1-year RCT.

Safety outcomes.   Safety was assessed by analysis of adverse events in both study medication groups and included laboratory investigations, physical examination, and electrocardiography.

Longer term follow-up

To evaluate whether the effect of levamisole remained constant or diminished over time and to analyze side effects of long-term levamisole treatment, patients in remission at trial completion and still receiving levamisole were followed for another 18 months (12 months receiving levamisole and 6 months without it).

Statistical analysis

Based on a meta-analysis a clinically relevant relative relapse risk of 0.60 during 1 year of levamisole treatment compared with placebo was expected (proportion of relapse with prednisone treatment alone 75% vs. 45% with levamisole).[25] Forty-two patients per treatment arm were sufficient to demonstrate such an effect, with a 2-sided significance level of 0.05 and a power of 80%. To allow for withdrawal (generally 10%–20%), we aimed to include 50 patients per arm.

The primary analysis population consisted of all patients who received at least 1 dose of the study medication (active or placebo) excluding 1 patient for whom information on the primary outcome was missing (i.e., modified intention-to-treat). The inclusion regions were compared regarding age (2–5, >5 years) and steroid dependence (yes, no) using the χ^2 test. Region was categorized as Europe and India because of the small number of patients included in France. A Kaplan-Meier survival curve was drawn to compare the primary outcome, time to relapse needing prednisone treatment in both treatment groups, and to check the proportional hazards assumption. A multivariable Cox proportional hazards analysis was performed to determine the effect of levamisole compared with placebo on time to relapse, expressed as a HR with a 95% CI, incorporating the stratification factors (SD, previous use of cyclophosphamide, region). In a secondary analysis, potential confounders (age, sex, and ethnicity) were tested. In addition, exploratory subgroup analyses were conducted to investigate effect modification of SD, region, sex, and age using treatment by subgroup interaction terms in the Cox proportional hazards model.

Only 6 patients with previous use of cyclophosphamide were included (Table 1), and therefore treatment effect modification by cyclophosphamide could not be adequately analyzed. Statistical tests of interaction terms directly examines the strength of evidence of the treatment difference varying between subgroups.[26,27] Patients who prematurely stopped the study medication due to an adverse event or other reasons were censored. Because the Kaplan-Meier curve showed that the proportional hazards assumption was not met, a time-dependent Cox regression analysis was conducted in which a time-dependent covariable was entered into the model as an effect modifier, and HRs were presented separately before and after 100 days post-randomization, the point at which the survival curves crossed.

For the occurrence of a prednisone-needing relapse, the effect estimate of levamisole versus placebo was calculated and expressed as a crude relative risk and an adjusted odds ratio after adjustment for the stratification variables using a logistic regression model. Statistical uncertainty of these estimates was expressed with 95% CIs.

A *P* value was obtained using a χ^2 test. Exploratory subgroup analyses were performed using formal tests of treatment by subgroup interaction terms in logistic regression models including the stratification variables.

All subgroup analyses should only be considered exploratory, yielding hypothesis-generating findings because the trial was not powered to adequately test subgroup effects and to allow adjustments for multiple testing related to the subgroup analyses.

The safety analysis was performed in the safety population (i.e., patients who received at least 1 dose of the study drug). A χ^2 or Fisher exact test was used as appropriate. Details on the safety analysis procedure are given in the Supplementary Material (item 1d).

All statistical analyses were performed with SPSS Statistics for Windows version 22 (IBM Corp., Armonk, NY).

SUPPLEMENTARY MATERIAL
Supplementary Material.
Table S1. Distribution of the lowest prednisone dose necessary to prevent a relapse in steroid-dependent patients at randomization.
Figure S1. Kaplan-Meier curve for relapse-free survival according to region subgroups (Europe and India). This figure shows the cumulative proportion of patients in the modified intention-to-treat population (mITT) according to region subgroups (Europe and India) staying in remission and on study medication over time from the start of the trial medication during a 1 year follow-up period of the RCT. Treatment by region interaction effect, *P* = 0.79 (see Results and Discussion section). Exploratory subgroup analyses were performed and statistically tested with interaction effects of the specific subgroup and treatment in Cox proportional hazards regression models including the stratification variables. Statistical tests of interaction terms directly examine the strength of evidence for the treatment difference varying between subgroups [1,2]. This subgroup analysis should only be considered exploratory, yielding hypothesis-generating findings since the trial was not adequately powered for subgroups analyses and to allow adjustments for multiple testing related to subgroup analyses.
Figure S2. Kaplan-Meier curves for relapse-free survival in patients with SD or FR. This figure shows the cumulative proportion of patients in the modified intention-to-treat population (mITT) in patients with steroid dependency (SD) and frequent relapse (FR). Treatment by steroid dependency interaction effect, *P* = 0.64 (see Results and Discussion section). Statistical tests of interaction terms directly examine the strength of evidence for the treatment difference

varying between subgroups [1,2]. Exploratory subgroup analyses were performed and statistically tested with interaction effects of the specific subgroup and treatment in Cox proportional hazards regression models including the stratification variables. This subgroup analysis should only be considered exploratory, yielding hypothesis-generating findings since the trial was not adequately powered for subgroups analyses and to allow adjustments for multiple testing related to subgroup analyses.

Table S2. Number of patients with a prednisone needing relapse during the 1 year follow-up period of the RCT according to exploratory subgroups in the modified intention-to-treat (miTT) population.

Figure S3. Glomerular filtration rate according to time. This figure shows the fraction of patients presenting with a GFR < 80 ml/min per 1.73 m^2 according to the method of Schwartz [3] at each RCT visit for levamisole (L) and placebo (P) groups. The number of patients assessed per visit is mentioned at the top of each column. The number of visits is included at the bottom of each column. A decreased GFR was observed at different times in the L group, but mainly at visit 3 (2 weeks after start of L). The lowest GFR observed was 52 ml/min per 1.73 m^2. Several low GFR values were observed during a relapse. In all other cases, no cause could be found, with spontaneous recovery occurring except in 1 patient, who recovered completely 5 days after L discontinuation for a GFR = 65.21 ml/min per 1.73 m^2.

Figure S4. Neutrophil values according to time. This figure shows the percent of patients with neutropenia (neutrophils < 1500/μl) at each RCT visit for levamisole (L) and placebo (P) groups. In the L group, neutropenia was observed in 7 patients, from whom 4 had 500–1000 neutrophils/μl, and 3 patients had 1000–1500 neutrophils/μl. Those values were distributed from visit 5 to 15 without any clear-cut temporal pattern.

Figure S5. Aspartate aminotransferase values according to time. This figure shows the percent of patients with high plasma values of aspartate aminotransferase (>40 IU/l) at each RCT visit for levamisole (L) and placebo (P) groups. High values have been observed at different moments in the L group, but mainly at visit 7. One patient with hepatitis B displayed values above 1000 IU/l. Several other patients presented moderately increased values, remaining under 100 IU/l.

Figure S6. The delta-height (height measured at visit 1[baseline] and visit 15 [1-year follow-up] in the levamisole and placebo group). This figure shows change in height (mean with 95% CI) between baseline (visit 1) and 1-year of follow-up (visit 15) in both study medication groups for patients who had a height measurement at both time points (placebo, n = 39; levamisole, n = 43). Delta height in levamisole verses the placebo group (mean, 95% CI): 6.86, 95% CI [6.03–7.69] versus 6.28, 95% CI [5.38–7.18], P = 0.88. Since 1-year height data were not available for all patients, this result needs to interpreted with caution and remains to be established in long-term studies.

Table S3. Summary of the levamisole trial protocol.

Table S4. Study visits.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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