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Population pharmacokinetics of levamisole in children with steroid-sensitive nephrotic syndrome

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WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT

- Levamisole reduces the frequency of relapses and reduces the steroid dose in children with steroid-sensitive nephrotic syndrome (SSNS).
- The pharmacokinetic profile of levamisole has been characterized in healthy subjects and cancer patients. However it has not been investigated in children with SSNS who relapse frequently.

WHAT THIS STUDY ADDS

- The pharmacokinetic profile of levamisole in children was similar to findings in adults, although the elimination rate was slightly higher in children.
- In addition to allometric scaling of the pharmacokinetic parameters, age had a significant effect on clearance.

AIM

The aim was to investigate the population pharmacokinetics of levamisole in children with steroid-sensitive nephrotic syndrome.

METHODS

Non-linear mixed effects modelling was performed on samples collected during a randomized controlled trial. Samples were collected from children who were receiving 2.5 mg kg^{-1} levamisole (or placebo) orally once every other day. One hundred and thirty-six plasma samples were collected from 38 children from India and Europe and included in the analysis. A one compartment model described the data well.

RESULTS

The apparent clearance rate (CL/*F*) and distribution volume (*V*/*F*) were 44 l h^{-1} 70 kg^{-1} and 236 l 70 kg^{-1}, respectively; estimated interindividual variability was 32–42%. In addition to allometric scaling of CL/*F* and *V*/*F* to body weight, we identified a significant proportional effect of age on CL/*F* (–10.1% per year). The pharmacokinetics parameters were not affected by gender, tablet strength or study centre. The median (interquartile range) maximum plasma concentration of levamisole was 438.3 (316.5–621.8) ng ml^{-1}, and the median area under the concentration–time curve was 2847 (2267–3761) ng ml^{-1} h. Median t_{max} and $t_{½}$ values were 1.65 (1.32–2.0) h and 2.60 (2.06–3.65) h, respectively.

CONCLUSIONS

Here, we present the first pharmacokinetic data regarding levamisole in children with steroid-sensitive nephrotic syndrome. The pharmacokinetic profile of levamisole in children was similar to findings reported in adults, although the elimination rate was slightly higher in children.

Introduction

Nephrotic syndrome (NS) is the most prevalent glomerular disease in children and is characterized by proteinuria, subsequent hypoalbuminaemia and oedema [1]. Moreover, children with NS have an increased risk of infection [2]. Although the majority of children with NS initially respond to corticosteroids (steroid-sensitive NS (SSNS)), most children with SNSS tend to relapse as soon as steroid therapy is discontinued or reduced [3]. However, repeated prolonged courses of steroids can cause steroid-induced side effects, including obesity, diabetes mellitus, growth failure, hypertension, infection and osteoporosis [1, 4].

Levamisole, a synthetic imidazothiazole derivative with immunomodulatory properties, is commonly used to reduce steroid usage. Levamisole has been used in the treatment of SSNS since 1980 [5]. Several studies found that levamisole reduces the frequency of relapses and reduces steroid dosage in patients with SSNS, both when used as a first alternative to steroids and after treatment failure with cyclophosphamide or ciclosporin [6–10]. However, the evidence obtained from these studies was rather limited, as only two of the five studies were randomized controlled trials [6, 8]. Trial quality was also inadequate, and the trials were small and relatively brief [11]. Importantly, these trials did not focus on the pharmacokinetics (PK) of levamisole in children. Finally, the dosages used in these studies (i.e. 2.5 mg kg^{-1} on alternating days and 2–3 mg kg^{-1} twice weekly) were extrapolated from other populations and indications, and little is known regarding the mechanism of action, PK and pharmacodynamics (PD) of levamisole in children with this specific indication.

In both animal models and human adults, the PK of levamisole have been investigated thoroughly. In healthy adults and adults with cancer or malaria, PK studies found that levamisole is rapidly absorbed by the gastrointestinal tract. Peak plasma concentrations reached 703–1030 ng ml^{-1} within 2 h of receiving a single oral dose of 150 mg or 2.5 mg kg^{-1} [12–17]. The reported elimination half-life of levamisole ranges from 4 h [17] to 5.6 h [14]. Moreover, high interindividual variability is observed. In one study, a seven-fold difference was found between the lowest and highest area under the concentration–time curve (AUC) values [13]. Less than 5% of the parent drug was recovered in its unchanged form in the urine of cancer patients who were receiving high dose levamisole treatment [17].

The PK profile of levamisole in children, particularly in children with NS, is currently unknown. In this study, plasma levamisole concentration was measured in children with nephrotic syndrome via sparse sampling. This study is part of a large, multicentre, randomized double-blind placebo-controlled clinical trial that is evaluating the efficacy and safety of levamisole. The traditional PK sampling method, which requires collecting multiple blood samples (*circa* 10–15 samples per subject), is considered ethically unacceptable in children [18]. Therefore, sparse sampling was used in this study. For this study, we used small, non-dividable film-coated tablets (5–8 mm in diameter) at four different strengths with identical dissolution profiles in order to provide long term treatment that is both flexible and accurate [19]. The objective of this study was to measure levamisole pharmacokinetics in children who were taking the above mentioned levamisole formulation to treat frequently relapsing steroid-sensitive nephrotic syndrome.

Methods

Study design

This pharmacokinetic study was conducted as part of a large, multicentre, randomized double-blind placebo-controlled trial (EudraCT number 2005-005745-18) to evaluate the efficacy of levamisole in preventing relapses (i.e. recurring proteinuria) in children with frequently relapsing SSNS [20].

The clinical results of this trial will be published separately. In the study, children (2–18 years of age) with SSNS were enrolled. The children were randomly assigned to receive either 2.5 mg kg^{-1} levamisole (or placebo) orally every other day for a period of 1 year. The study protocol and the informed consent procedures were reviewed and approved by the Ethics Committee of each participating centre prior to the start of any study-related procedures. Written informed consent was obtained from each patient (or legal representative) before the patient entered the study.

The selection criteria included patients 2–18 years of age who were diagnosed with frequently relapsing idiopathic SSNS either with or without steroid dependency. Idiopathic NS was defined as the presence of proteinuria (urinary protein $>$200 mg $mmol^{-1}$ creatinine) and hypoalbuminaemia (serum albumin $<$25 g l^{-1}) with no signs of any specific aetiology such as Henoch–Schönlein purpura or acute post-infectious glomerulonephritis. NS was classified as steroid-sensitive when steroid treatment induced remission. Frequent relapses were defined as the occurrence of ≥two relapses within 6 months of the initial response or ≥four relapses in any 12 month period.

Remission was defined as proteinuria levels $<$20 mg $mmol^{-1}$ creatinine (or at most a trace amount of protein on a dipstick test) for ≥3 consecutive days. A relapse was defined as recurring proteinuria (dipstick 3+ or proteinuria $>$200 mg $mmol^{-1}$ creatinine) after remission for 3 consecutive days.

The following exclusion criteria were applied: previous treatment with levamisole, non-responsiveness to ciclosporin or mycophenolate mofetil (MMF, CellCept®), concomitant immunosuppressive medication (ciclosporin,

cyclophosphamide, MMF and/or other immunosuppressive drugs), nephrotic syndrome due to a specific kidney disease (e.g. Henoch–Schönlein purpura, acute infectious glomerulonephritis, lupus erythematosus, or kidney disease associated with hepatitis B or C), the presence of neutropenia, convulsions, hepatic disease, and/or prolonged corrected QT interval on surface electrocardiography (>0.44 s) at presentation and pregnancy, breast-feeding or planned pregnancy during the study.

Drug dosage and administration

Small non-dividable film-coated levamisole tablets (5–8 mm in diameter) were manufactured in four strengths (5, 10, 25 and 50 mg) to provide dosing that is both flexible and accurate [19]. Levamisole was administered based on total body weight at a dosage of 2.5 mg kg^{-1} every other day, with a maximum daily dose of 150 mg. To determine the appropriate posology for the study patients (while allowing only one strength per intake, to minimize dosing errors), a dosage schedule was established [19].

Patients who presented with a relapse were treated with prednisone or prednisolone (60 mg m^{-2} once daily) until remission was achieved. After the urine was essentially protein free for 3 to 21 days, levamisole (or placebo) was started, and corticosteroids were gradually reduced over the next 4 months in accordance with a previously described corticosteroid scheme [21]. Treatment was discontinued after 12 months or when a relapse that necessitated prednisone or prednisolone treatment occurred. Levamisole-treated patients who were still in remission at trial completion continued taking levamisole for an additional 12 months (cohort phase).

PK sample collection

To minimize the volume of blood obtained from our paediatric cohort, PK samples were obtained by sparse sampling during four regular visits (in weeks 8, 12, 20 and 24), with a maximum of six samples per patient. This sampling regimen was based on advice from the Scientific Advice Working Party of the European Medicines Agency (EMA). In weeks 8 and 20, one blood sample was taken shortly before medication intake (i.e. pre-dose, C_{min}), and one post-dose sample was taken at the estimated peak plasma concentration (C_{max}, 1, 2, 4 or 6 h after drug administration. The time point was determined by computerized randomization). Patients were instructed to fast prior to the collection of the pre-dose blood samples. In weeks 12 and 24, one plasma sample was taken on a medication free day (i.e. a 24 h post-dose sample).

The precise times of sampling and drug administration were recorded. Blood samples were collected in plastic tubes (Vacuette LH Lithium Heparin, 3 ml; Ref. 454244, Greiner Bio-One GmbH, Kremsmünster, Austria) and centrifuged within 30 min at 2000 *g* for 15 min. The plasma fraction was removed and stored at −20 °C. Samples obtained from all study sites were shipped on dry ice to the contract laboratory Analytical Biochemical Laboratory (ABL BV, Assen, the Netherlands), where they were stored at −70 °C until analysis.

Bioanalytical assay

The levamisole concentration in the plasma samples obtained from the study subjects was measured using a liquid chromatography-tandem mass spectrometry (LC-MS/MS) system as developed and validated by ABL in collaboration with ACE Pharmaceuticals. The assay was based on the liquid–liquid extraction of analytes from human plasma using diethyl-ether after addition of the internal standard levamisole D5. Chromatographic separation was performed through a PFP Kinetix column (100 mm × 3.0 mm, 2.6 μm, Phenomenex Inc., Torrance, CA, USA) at 50 °C, with a mobile phase consisting of 2.5 mm ammonium acetate : acetonitrile (35 : 65, v/v). The flow rate was 1.0 ml min^{-1}, and the total run time was 4 min. Detection and quantification were performed by mass spectrometry using an API 4000 tandem mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) equipped with a turbo-ion spray operated in positive mode for the multiple reaction monitoring.

The LC-MS/MS method used to measure levamisole in human heparin-containing plasma samples was validated using current guidelines for validating bioanalytical methods [22–24]. Both the overall accuracy (expressed as % bias) and the precision (expressed as the coefficient of variation (%CV)) of the calibration curve were within 15% of the target value at every concentration. The within-run and between-run accuracy bias and precision CV values were also <15%. The assay was linear over a concentration range of 5–2000 ng ml^{-1} and the lower limit of quantification was 5 ng ml^{-1}. The overall analytical recovery of levamisole was 89.4%, which was similar to the overall recovery of the internal standard (90.1%). Levamisole was stable for 206 and 486 days when stored below −20 °C or at −70 °C, respectively. Our analysis revealed that the LC-MS/MS method used to measure levamisole in human heparin-containing plasma samples was both accurate and precise within the analytical range of 5–2000 ng ml^{-1} (data not shown).

PK data analysis

The plasma levamisole concentrations were analyzed in a population approach using non-linear mixed effects modelling. Plasma samples were collected from each subject during four visits. However, because most of the plasma samples taken ≥24 h post-dose (i.e. the pre-dose samples taken in weeks 8 and 20 and the 24 h post-dose samples taken in weeks 12 and 24) were below the lower limit of detection (102 out of 121 samples), all of the data obtained at these sampling time points were deemed uninformative for this PK model and were therefore excluded from the final PK dataset, thereby

preventing any potential bias in our estimation of clearance rate. All estimations and calculations were performed with a dual-core Intel Core i7 processor running Mac OSX 10.9.1 with the software program nonmem 7.3 (with FORTRAN compiler gfortran 4.9). The program R (version 3.0) was used to manage and clean the data. Perl-speaks-nonmem (PsN version 4.2.0) was used for automated stepwise covariate analysis and data generation for the visual predictive check (VPC), which was created using the R package Xpose (version 4.4.0). Pirana (version 2.9.0) was used for run deployment, for basic diagnostics, and as an interface between R, PsN, and Xpose.

Based on the bioanalytical validation report, storage time had a linear effect on the stability of the samples, with an average decrease in stability of 0.05% per day in storage (validated up to 486 days). Therefore, storage duration was included in the model as a fixed effect (0.0005 × days in storage at –20 °C) directly on the observed concentrations, and the model was evaluated using the storage time-corrected observed concentrations.

Non-linear mixed effects modelling was performed with nonmem using the first order conditional estimation procedure, including interaction between interindividual variability and residual error components.

Interindividual variability in the PK parameters was estimated using an exponential model. For example, variability with respect to clearance was described by the equation $[CL/F_i = \theta_1 \times \exp(\eta_i)]$, where CL/F_i represents clearance of the i^{th} individual, θ_1 is the typical clearance value and η_i is the interindividual random effect with a mean of 0 and a variance of ω^2. Because the interindividual variability of *V*/*F* correlated 100% with the interindividual variability of CL/*F* (resulting in overparameterization of our model), the interindividual variability for CL/*F* was estimated and used to estimated *V*/*F* using an additional scaling parameter. Residual variability was modelled using a proportional error model. Single-compartment and multi-compartmental models (with first order absorption and linear elimination from the central compartment) were evaluated. The primary PK parameters estimated included the absorption rate constant (k_a), clearance (CL) and volume of distribution (*V*). In addition, absorption lag time was evaluated as well since this was suggested in a previous publication [13]. Because bioavailability (*F*) was unknown, parameters were estimated relative to bioavailability (CL/*F* and *V*/*F*). Goodness-of-fit was guided by the objective function value (OFV, equal to –2*log-likelihood) and assessed further with basic goodness-of-fit plots and a VPC.

An automated stepwise covariate analysis was performed using PsN, with *P* value thresholds of 0.05 and 0.01 for the forward-inclusion and backward-elimination steps, respectively. In addition to body weight (which was already included in the base model), the following covariates were evaluated (the parameters on which they were evaluated are given in parentheses): age (CL/*F*, *V*/*F*; continuous), gender (CL/*F*, *V*/*F*, k_a; categorical), inclusion in a European centre/Indian centre (CL/*F*, *V*/*F*, k_a, *F*; categorical) and tablet strength (CL/*F*, *V*/*F*, k_a, *F*; categorical). To reduce the likelihood of obtaining false positive covariate–parameter relations, a selective covariate analysis was performed. All covariates were tested on CL/*F* and *V*/*F*. In addition, inclusion in a European centre/Indian centre and tablet strength were tested on *F* to account for the type of food intake (which varied the most between India and Europe) and dissolution of the various tablet formulations, respectively. Gender was tested on k_a, as this relationship was reported in a previous study [14]. Secondary PK parameters, including elimination half-life ($t_{1/2}$), C_{max}, t_{max} and AUC, were estimated for each parameter and for each subject from the population model using the empirical Bayes estimation method.

Results

The final dataset contained 136 plasma levamisole concentrations obtained from 38 individuals (27 males and 11 females) with a median body weight of 21 kg (range 11–68 kg) and a median age of 6.28 years (range 2.35–13.10 years). The mean levamisole dose was 2.45 mg kg^{-1} every other day based on a previously described dosing schedule [19]. The patient characteristics are summarized in Table 1, and the observed (prediction-corrected) plasma concentrations are shown in Figure 1.

A one compartment model with first order absorption and first order elimination from the central compartment

Table 1

Patient characteristics at inclusion

Characteristic	Value
n **(male/female)**	38 (27/11)
Median age (range), years	6.28 (2.35–13.10)
Median weight (range), kg	21 (11–68)
Country, *n* (%)	
India	18 (47.4)
The Netherlands	6 (15.8)
Belgium	5 (13.2)
France	4 (10.5)
Poland	4 (10.5)
Italy	1 (2.6)
Ethnicity, *n* (%)	
Caucasian	18 (47.4)
Asian	19 (50)
Unknown	1 (2.6)
Mean plasma creatinine (SD), μmol l^{-1}	30.0 (10.4)
Median urine protein concentration* (range), g l^{-1}	0.17 (0.0-23.70)
Mean levamisole dose (SD), mg kg^{-1} every other day	2.45 (0.24)

*Urine sample quantitative protein after start of prednisone but before start study medication, which was started when the urine was protein-free for 3 to 21 days.

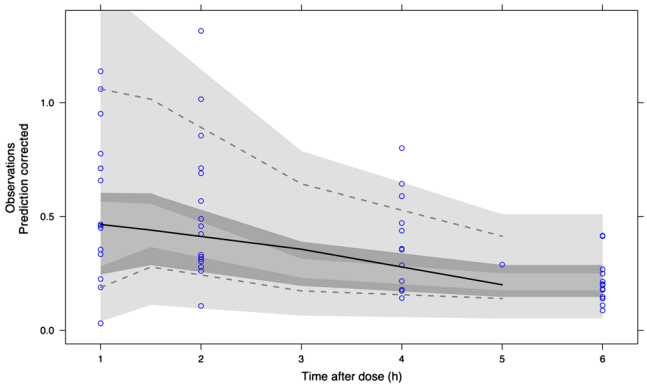

Figure 1

Prediction-corrected visual predictive check of the final model for the observed levamisole concentrations, based on 1000 Monte Carlo simulations to assess the predictive performance of the model. Prediction-corrected simulated (areas) and observed (circles and lines) levamisole concentrations are shown *vs.* time after dose. Simulations are represented as the non-parametric 95% confidence interval of the simulated 50th (dark grey area), 5th and 95th (light grey areas) percentiles. For the observed values, the 50th (continuous line), 5th and 95th (broken lines) percentiles are depicted

fitted the data best. Because samples were not collected during the absorption phase (i.e. within 1 h of dosing), and because the model did not converge successfully with an estimated k_a after covariates were included, in the final model the typical value for k_a was fixed to a value estimated prior to the covariate analysis (1.2 h^{-1}), while allowing for the between subject variability of this parameter. A sensitivity analysis using various k_a values did not reveal any strong effect of the actual value of k_a on the overall fit of the model. The applicability of an absorption lag time was also evaluated, as this was proposed in a previous population PK study of levamisole [13]. However, the inclusion of either the fixed or estimated lag times did not improve the fit, likely because insufficient data were available from the absorption phase (i.e. no samples were collected within 1 h of dosing) and because it is mechanistically highly implausible. Plasma samples from a given individual were collected during different visits. Therefore, to account for potential differences in bioavailability between visits, we evaluated the applicability of between occasion variability on bioavailability. However, due to the sparseness of the data, between occasion variability could not be distinguished from residual variability and thus could not be estimated reliably.

The parameter estimates from the population model are summarized in Table 2. Allometric scaling of CL/*F* (power value of 0.75) to standard body weight (70 kg) was evaluated and yielded a better fit of the model than linear scaling (corresponding allometric power of 1) of CL/*F* and *V*/*F* (Table 3). Shrinkage of the interindividual variability for CL, *V* and k_a was 17%, 17% and 44%, respectively, and shrinkage of the residual variability was 36%. All of these values are relatively modest given the sparseness of the data.

Table 2

Population parameter estimates of the levamisole population PK model. CL/*F* and *V*/*F* were normalized to a standard body weight of 70 kg and age effect on CL/*F* was normalized to the population median age of 6.28 years (the median value in our population). Parameter precisions were derived from the covariance step in nonmem.

Parameter	Estimate (RSE [%])	% Interindividual variability(RSE [%])
CL/*F* (l h^{-1} 70 kg^{-1})	44 (8.5)	31.6 (35.9)
V/*F* (l 70 kg^{-1})	236 (13.3)	41.7 (25.5)
k_a (h^{-1})	1.2 (fixed)	92.2 (44.7)
Proportional residual variability (%)	20.7 (25.4)	Not estimated
Proportional change in CL/*F* per life year (%/year)	–10.1 (25)	Not estimated

RSE, relative standard error.

Table 3

Effect of parameter scaling and covariate inclusion on model fit and interindividual variability.

Model	OFV	ΔOFV	Interindividual variability CL/*F*	Interindividual variability *V*/*F*
1. Linear scaling of CL/*F* and *V*/*F* to BW (70 kg^{-1})	–133.6	0	49.8%	48.6%
2. Allometric scaling of CL/*F* and *V*/*F* to BW (70 kg^{-1})	–138.6	–4.96*	43%	46.1%
3. Model 2 + inclusion of additional covariate age on CL/*F*	–146.6	–13.03**	31.6%	41.7%

BW, body weight; OFV, objective function value; *$P < 0.05$; **$P < 0.01$ (compared with the first model).

The stepwise covariate analysis revealed only an additional significant effect of age on CL/*F* (described with a linear function) with respect to the fit of the model. The effect of including covariates on the fit of the model and the interindividual variability is shown in Table 3. The model shows a proportional decrease in CL/*F* with increasing age in addition to the allometrically scaled effect of body weight, as shown in the base model: $[CL/F_i = \theta_{CL/F} \times - 0.101 \times (age - 6.28) \times (weight/70)^{0.75}]$, where $\theta_{CL/F}$ is the typical value for CL/*F* and 6.28 is the median age in our population. CL/*F* was also plotted against age, and the results are provided in Figure S1. Adding age as a covariate on CL/*F* caused a significant decrease in OFV, as well as a clinically relevant decrease in interindividual variability with respect to both CL/*F* (–11.4%) and *V*/*F* (–4.1%, Table 3. The other covariates tested did not improve the model and therefore were not included in the final model.

A VPC of the model is shown in Figure 1 and basic goodness-of-fit plots are shown in Figure 2. Neither the goodness-of-fit plots nor the prediction-corrected VPC

showed any deviating trend and indicated a good fit of the model to the data, given that the 5th, 50th and 95th percentiles were all within the simulated confidence intervals.

Table 4 summarizes the secondary PK parameters derived from the individual Empirical Bayes Estimation values of the parameters.

Discussion

In this study, we evaluated the population pharmacokinetics of levamisole in a cohort of children with SSNS. The PK

Table 4

Secondary PK parameter estimates derived from the individual model-based empirical Bayes estimates obtained from the population PK model. AUC(0,∞) represents the AUC after a single dose of levamisole.

Secondary parameter	Median	Interquartile range
t_{max} (h)	1.65	1.316–1.954
C_{max} (ng ml^{-1})	438.3	316.5–621.8
AUC(0,∞) (ng ml^{-1} h)	2847	2267–3761
$t_{1/2}$ (h)	2.60	2.06–3.65

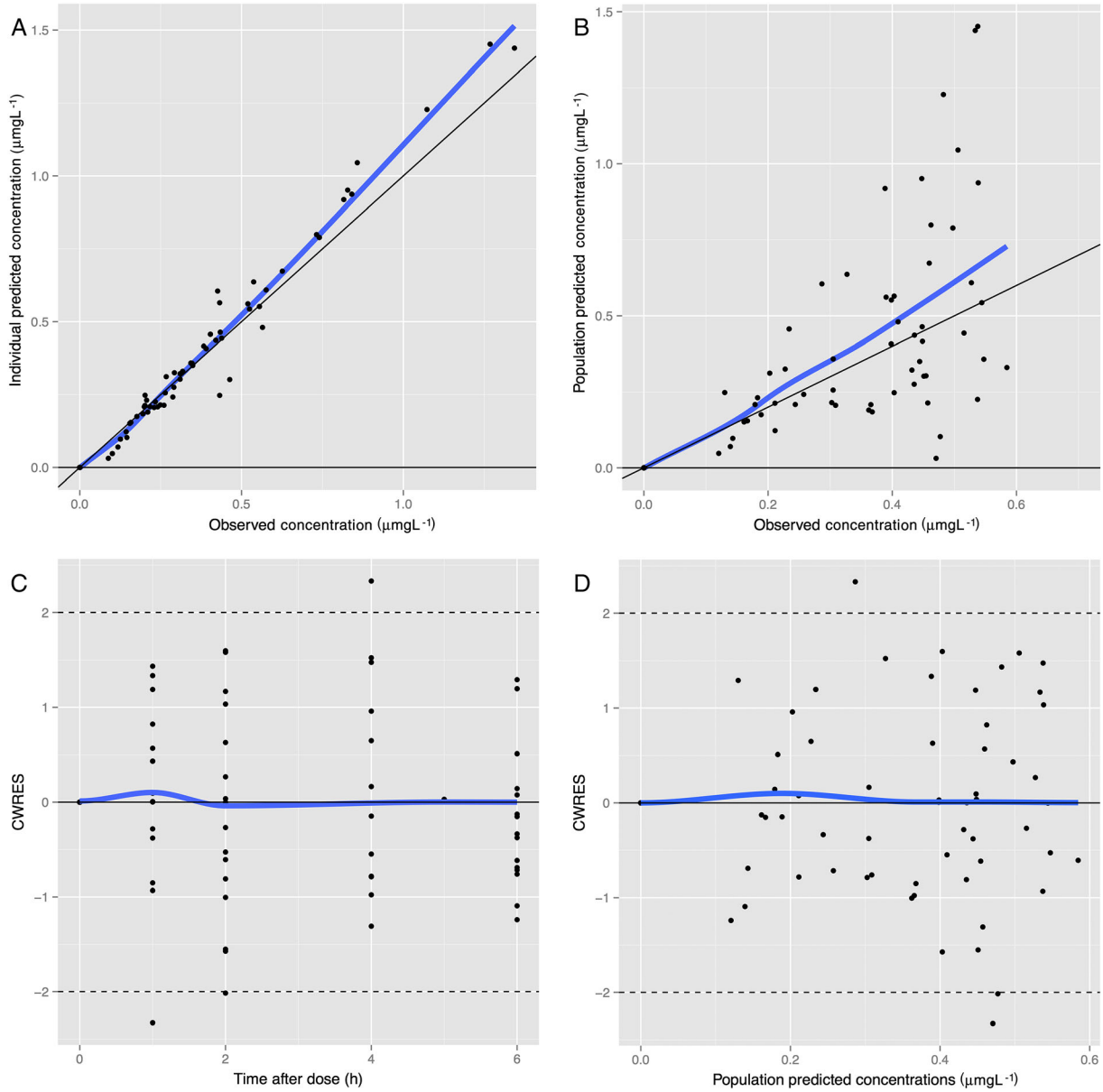

Figure 2

Basic goodness-of-fit plots of the final levamisole population PK model. A) Individual predicted concentration *vs*. observed concentration, B) population predicted concentration *vs*. observed concentration, C) CWRES *vs*. time after dose and D) CWRES *vs*. population predicted concentrations. CWRES conditional weighted residuals. The black lines indicate the unit line or the line of identity. The thick blue line is a smooth line showing the trend in the observations

data were fitted best using a one compartment model with first order elimination. The results revealed moderate interindividual variability with respect to CL/*F* and *V*/*F*. A stepwise covariate analysis identified an effect of age on CL/*F* in addition to allometric scaling of CL/*F* and *V*/*F* by body weight. In contrast, the PK parameters were not affected by gender, tablet strength or study centre.

This is the first analysis of levamisole pharmacokinetics in a paediatric population. Because traditional PK sampling (with rich datasets containing data from multiple samples per subject) is not suitable for children [18], we used sparse sampling and population PK analysis. Population-based modelling is an elegant and effective way to analyze limited and/or sparsely collected data [18] and is recommended by the EMA for paediatric PK studies [25]. In our study, levamisole concentration–time profiles were studied in a cohort of children with a wide age range (2.4–13.1 years) and a wide body weight range (11–68 kg), thereby providing a more reliable prediction of PK profiles in individual patients than if a narrow age range had been used. A limitation of this study was the time interval between sampling and analysis of the PK samples. This prolonged interval was due to the fact that NS is a relatively rare entity (with a prevalence of 2–7 cases per 100 000 individuals), which required a long trial enrolment period. For the majority of the collected samples, storage time exceeded the established stability of 206 days, after which the concentration in the plasma decreases linearly. This decrease, however, was consistent within a given patient, as samples were taken within a 4 month period from each patient. Although most studies do not routinely account for storage time in their analyses, in our study we accounted for this factor by introducing storage time as a fixed effect in the final model.

The CL/*F* and *V*/*F* values that were estimated based on the standard body weight (70 kg) of our paediatric population were similar to values reported for both healthy adults and adults with cancer [12, 14, 15,17, 26]. The elimination half-life was faster (2.6 h) in our paediatric cohort than in adults, in whom elimination half-life ranged from 4 h [17] to 5.6 h [14], with a recently described shorter elimination half-life of 2 h in a healthy adult volunteer receiving one single oral dose of 100 mg levamisole [26]. The faster elimination in our paediatric cohort resulted in shorter exposure of levamisole compared with adults, particularly with respect to C_{max}, which was 0.44 mg l^{-1} *vs*. 0.7-1.0 mg l^{-1}, respectively [12, 14–17].

Differences with respect to results between studies can be explained largely by the different bioanalytical methods that are used to detect levamisole in human samples [27] and the methods used to estimate PK parameters [13, 28, 29]. The LC-MS/MS system that we used is similar to systems used by other groups with respect to recovery, accuracy and precision [30–32]. Furthermore, the non-linear mixed effects modelling approach that we used in our study is preferred over naïve pooled data and the two stage approach, as our approach can pool data while still taking into account interindividual and intra-individual variability, thereby yielding estimates that are more precise and more accurate [33].The median AUC(0,∞) for our population was well within the lowest and highest AUC values reported in another study that used a population approach [13].

Importantly, we found moderate interindividual variability in our population, which is consistent with the variability reported for healthy adults and adults with cancer who were taking similar doses of levamisole [13–15]. The sources of this variability are poorly understood. However, weight and cardiovascular, gastrointestinal, and liver complications can alter levamisole pharmacokinetics [13–15]. In addition to the general effect of allometric scaling of CL/*F* and *V*/*F* to body weight, we found that age had a significant declining effect on CL/*F*. The magnitude of the effect of age on CL/*F* can be subject to many factors, including degradation during storage. However, the effect itself may be explained by age-dependent activity of drug-metabolizing enzymes (DMEs). Although the specific DMEs that metabolize levamisole in humans have not been identified [34], levamisole is metabolized extensively in the liver, where it undergoes P450-mediated α-carbon hydroxylation, oxidation, desulfurization, N-dealkylation, S-methylation and/or sulphoxidation [35]. In general, DME activity peaks in early childhood and declines thereafter [36, 37]. This declining enzyme activity increases clearance and reduces the drug's first pass effect. Other age-dependent changes (for example, drug bioavailability) seem unlikely. Although age groups differ with respect to gastric emptying, intestinal transit time, pancreatic enzyme activity and colonization composition and rate, these differences generally do not affect the rate or extent of drug absorption [38]. PK parameters can also be affected by age-related changes in distribution, particularly age-dependent factors such as protein binding, body compartment size and composition, haemodynamic factors, membrane permeability and the drug's physiochemical properties (e.g. fat and/or water solubility) [39]. On the other hand, age-dependent changes in renal clearance do not appear to play an important role in PK, as glomerular filtration rate is relatively constant between the ages of 18 months and 16 years [40]. However, any age-dependent effect should be interpreted with great care, as the extent of such an effect is likely specific to the ages included in our study.

In contrast to a previous study [14], we found no gender-related differences. However, we believe that the apparent gender-based differences in adults can likely be attributed to differences in body size and/or composition between men and women (rather than to gender *per se*). In addition, we found no difference between study centres. Other covariates that can affect pharmacokinetics include body surface area, body mass

index, disease characteristics (e.g. glomerular filtration rate, liver enzyme levels and white blood cell count), interactions with other medications and diet. These covariates were not available for our cohort and were therefore not analyzed. Although information regarding food intake was recorded during blood sampling, the subjects were not placed on a standardized diet. Therefore, any differences in dietary habits between countries precluded an assessment of diet on our analysis of PK.

The administration of various tablet strengths (5, 10, 25 or 50 mg levamisole tablets) did not affect the PK parameters in our population. This finding is consistent with previous reports in which dissolution rates were similar for all four tablet strengths [19]. Furthermore, both the rate and extent of dissolution were independent of pH. This is an important point, as gastrointestinal transit time and intraluminal pH can vary greatly in young children [41]. Because all four tablet strengths have essentially the same composition and release pattern, different strengths can be combined unrestrained without compromising drug availability while allowing for minute dose adjustments. The ability to adjust the dose over time is important, given that patients with nephrotic syndrome are usually treated for a long period of time, during which their changing age and body weight may require dose adjustments during the treatment period.

The therapeutic range of levamisole has not been determined precisely. However, levamisole-induced toxicity in NS therapy is relatively rare and generally reversible. Toxicity can include serious haematological complications such as neutropenia [42–44], leukopenia [45], thrombocytopenia [46] and cutaneous reactions [47, 48]. Rare neurological complications, including convulsions [49] and ataxia [50], have also been reported. In rare cases, cutaneous or disseminated vasculitis [51, 52] can occur, although this usually resolves without sequelae after levamisole treatment is discontinued. With respect to children, neither a dose–exposure relationship nor a maximum tolerable dose (MTD) has been established. The recommended dose of levamisole for children with SSNS is 2.5 mg kg^{-1} every other day, although a range of 2–3 mg kg^{-1} every other day has also been used [53]. For adult cancer patients who are also being treated with 5-fluorouracil for 5 consecutive days, the MTD of levamisole was 100 mg m^{-2} (approximately 2.7 mg kg^{-1}) [17], with peak plasma concentration ranging from 0.6 to 1.13 μg ml^{-1}. In children weighing 20 kg, an MTD of 100 mg m^{-2} is equivalent to 4 mg kg^{-1}, which is considerably higher than the dose used in this study. Consequently, the peak plasma concentration reached in our population was considerably lower than in adult cancer patients. Although a precise dose–response relationship remains to be established, our data suggest that the administered dose was within the therapeutic range.

The mechanism through which levamisole exerts its effect, presumed modulation of immune response, is not completely understood. However, the biological half-life of levamisole is apparently much longer than the plasma elimination half-life. The short elimination half-life (<3 h) is in contrast with the efficacy observed when dosing on alternate days. This contrast may be explained by a considerably longer biological half-life and/or a prolonged effect of the two principal metabolites of levamisole, p-hydroxylevamisole and aminorex [12]. In our study, it was not possible to measure these metabolites at appropriate concentrations in the blood. It is likely that the biological effect is indirect, by restoring impaired cell mediated immunity towards a type 1 response [54–56]. The relationship between plasma levamisole concentration and the effect of preventing relapse of proteinuria remains unclear. We have to await the PD results which are in progress.

In conclusion, a one compartment model with first order absorption and first order elimination from the central compartment adequately described the plasma concentration–time curve of levamisole in children with SSNS. In addition to allometric scaling to body weight, age was identified as a covariate, with a significant effect on CL/*F*. The PK parameters were similar to parameters measured in healthy adults and cancer patients treated with levamisole, although the elimination rate was slightly faster in children than in adults, resulting in lower exposure in children. Finally, an exposure–response relationship should be established in children with SSNS.

Authors' Contributions

MG conceived the study, participated in the study design, and coordinated and supervised the clinical study. AKV performed the pharmacokinetics study, participated in the analysis and interpretation of the data and drafted the manuscript. TD performed the statistical analysis, interpreted the data and helped draft the manuscript. PJdV interpreted the data and helped draft the manuscript. AdB helped draft the manuscript. All authors have read and approved the final manuscript.

PI statement

The Principal Investigator of the levamisole trial will author the primary manuscript detailing clinical efficacy and safety data (manuscript in preparation) and did not participate in the pharmacokinetic study design, data analysis and interpretation. The authors of the current manuscript were involved in the pharmacokinetic study design, data analysis, and interpretation of the data during the levamisole trial.

Competing Interests

All authors have completed the Unified Competing Interest form at http://www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare that A.R. Kreeftmeijer-Vegter and P.J. de Vries report personal fees from ACE Pharmaceuticals received during the conduct of the study. The remaining authors have nothing to disclose. The double-blind, placebo-controlled, randomized, multicentre levamisole trial was funded by the Dutch Kidney Foundation. The development, manufacture, and supply of levamisole tablets was performed by ACE Pharmaceuticals BV, Zeewolde, the Netherlands, who also provided funding for the pharmacokinetic part of the study.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1
Plot showing apparent clearance (Cl/F) versus age