High dose genistein in Sanfilippo syndrome: A randomised controlled trial

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Abstract
The aim of this study was to evaluate the efficacy of high dose genistein aglycone in Sanfilippo syndrome (mucopolysaccharidosis type III). High doses of genistein aglycone have been shown to correct neuropathology and hyperactive behaviour in mice, but efficacy in humans is uncertain. This was a single centre, double-blinded, randomised, placebo-controlled study with open-label extension phase. Randomised participants received either 160 mg/kg/day genistein aglycone or placebo for 12 months; subsequently all participants received genistein for 12 months. The primary outcome measure was the change in heparan sulfate concentration in cerebrospinal fluid (CSF), with secondary outcome measures including heparan sulfate in plasma and urine, total glycosaminoglycans in urine, cognitive and adaptive behaviour scores, quality of life measures and actigraphy. Twenty-one participants were randomised and 20 completed the placebo-controlled phase. After 12 months of treatment, the CSF heparan sulfate concentration was 5.5% lower in the genistein group (adjusted for baseline values), but this was not statistically significant ($P = .26$), and CSF heparan sulfate increased in both groups during the
open-label extension phase. Reduction of urinary glycosaminoglycans was significantly greater in the genistein group (32.1% lower than placebo after 12 months, $P = .0495$). Other biochemical and clinical parameters showed no significant differences between groups. High dose genistein aglycone (160 mg/kg/day) was not associated with clinically meaningful reductions in CSF heparan sulfate and no evidence of clinical efficacy was detected. However, there was a statistically significant reduction in urine glycosaminoglycans. These data do not support the use of genistein aglycone therapy in mucopolysaccharidosis type III. High dose genistein aglycone does not lead to clinically meaningful reductions in biomarkers or improvement in neuropsychological outcomes in mucopolysaccharidosis type III.

KEYWORDS

genistein, lysosomal storage disorders, mucopolysaccharidosis, Sanfilippo, substrate reduction therapy
June 2018 at Manchester University Hospitals Foundation Trust. The study was registered in clinical trials database EudraCT: 2013-001479-18. Ethical approval was given by the Office for Research Ethics Committees, Northern Ireland (ORECNI) (REC 14/NI/0006). Informed consent was obtained for all participants and the study was conducted in accordance with the principles set out in the WMA Declaration of Helsinki.

2.2 | Clinical trial design

This was a single-centre, randomised, double-blinded, placebo-controlled trial with open-label extension. Participants were randomised to receive either placebo or 160 mg/kg/day genistein aglycone for 12 months. All participants subsequently received 160 mg/kg/day genistein aglycone for a further 12 months. The primary outcome measure was HS concentration in cerebrospinal fluid (CSF) at 12 months. Secondary outcome measures were: CSF and plasma genistein levels, urine and plasma HS, total urinary GAG, neuropsychological assessments, actigraphy, caregiver mood, and quality of life measures. Safety assessments included physical examinations (including pubertal staging), adverse event (AEs) recording, six-monthly monitoring of haematological and biochemical parameters in blood (including hepatic profile, thyroid function tests, gonadotropin levels and sex hormones), and six-monthly ultrasound examination of the pelvis in female participants.

2.3 | Participants

Inclusion criteria were: (a) aged between 2 and 15 years (inclusive) at the time of consent; (b) confirmed diagnosis of MPS IIIA, B or C by enzyme assay in leukocytes or fibroblasts, and/or presence of known pathogenic mutations in SGSH, NAGLU or HGSNAT; (c) clinical signs and symptoms of MPS III; (d) able to walk unaided. Exclusion criteria were: (a) previous haematopoietic stem cell transplantation; (b) use of genistein or any other investigational therapy for MPS III; (c) known adverse reaction to genistein; (d) pregnancy; (e) clinically significant symptoms unrelated to MPS III that would influence results in the opinion of the investigators.

2.4 | Sample size calculation

An indirect sample size estimate was made on the basis of uGAG reduction observed in a previous placebo-controlled crossover study of genistein. As a much higher dose of genistein was used in this study, a difference of twice that seen in the de Ruijter et al study was targeted. A sample size of 24 participants was estimated to have 90% power to detect such a difference. A total of 21 participants were recruited; 20 completed the placebo-controlled phase and were included in the analysis (Figure 1).

2.5 | Early termination of study

The study was terminated in June 2018 due to shelf-life expiry of the genistein aglycone product. All active participants attended 24-month study visits prior to study termination. CSF samples were not obtained for two participants due to logistical difficulties, but all other study procedures were completed.

2.6 | Interventions

Genistein aglycone powder (BONISTEIN) was manufactured and tested according to good manufacturing practice (GMP) standards by DSM Nutritional Products Ltd and was re-packaged into 250 g (210 mm × 135 mm) laminated foil pouches by Quay Pharmaceuticals, also at GMP standard. Matching placebo product (Maize starch 99.7/quinolone yellow 0.3%) was manufactured by Quay Pharmaceuticals and packaged in identical pouches. A stability testing programme was implemented according to ICH stability guidelines, 6 months following re-packaging of the Genistein, and annually thereafter for the duration of the trial. These tests were performed by DSM Nutritional products Ltd and CoAs issued to comply with GCP and GMP. Blind testing was implemented to ensure matching of placebo and drug.

The dose of genistein aglycone for each individual was converted into a specified number of standardised scoops (1.25 mL or 2.5 mL) of powder. A scoop validation study was performed with caregivers (n = 4) to assess accuracy of dosing with a maximum variation (95% CI) of −1.95% to +13.9% for a target dose of 3.0 g (6 × 1.25 mL scoops) and 95% CI of +4.0% to +8.5% for a target dose of 6.0 g (6 × 2.5 mL scoops). 95% of measurements were within 13.5% tolerance for the 1.25 mL scoop and 95% within 8.5% tolerance of the expected dose for the 2.5 mL scoop. Caregivers were instructed to give a specified number of scoops of powder, and instructions were updated at each study visit based on participant weight. Details of frequency of missed doses were recorded at each study visit to estimate compliance.

2.7 | Randomisation

Participants were randomised in a 1:1 ratio to receive genistein or placebo, stratified by MPS subtype (MPS IIIC

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vs MPS IIIA or IIIB) and with allocation in blocks of random size. Randomisation was performed using an online randomisation tool (www.randomisation.com). All participants, caregivers, intervention providers, investigators and statisticians remained blinded until the end of the placebo-controlled phase, following which the allocation list was revealed to investigators.

### 2.8 Outcome measures

#### 2.8.1 Biochemical outcome measures

HS concentrations in CSF, plasma and urine were measured at Amsterdam UMC using previously described methods. In short, samples were prepared by heparinase digest and resultant disaccharides were quantitated by UPLC-MS/MS. Total HS was calculated as the sum of D0A0, D0S0, D0A6 + D2A0, and D0S6 + D2S0 disaccharides. Urine HS measurements were corrected for creatinine concentration. Urinary GAG concentrations were measured using the dimethylmethylen blue (DMB) assay at the Willink Biochemical Genetics Unit, Manchester.

#### 2.8.2 Neuropsychological evaluations

Neuropsychological evaluations were performed by specialist paediatric neuropsychologists with substantial experience of assessing children with MPSIII. Cognitive assessment was performed using age-appropriate tests: for most participants, the Bayley Scales of Infant and Toddler Development, Third Edition (BSID-III) were used. The Stanford-Binet Intelligence Scales, Fifth Edition was used in one participant. Adaptive behaviour was assessed using the Vineland Adaptive Behaviour Scale—Survey Interview Form, Second Edition (VABS-II). Scores are expressed as age equivalent scores (AgeEqSs) and developmental quotients (DQs), where $DQ = \frac{\text{AgeEqSs}}{\text{Chronological Age}} \times 100$.

#### 2.8.3 Caregiver mood and quality of life measures

Caregivers completed the Beck Depression Inventory (BDI-II), a self-report inventory for measuring the severity of depression. Scores range from 0 to 63 and defined as: 0 to 13, minimal depression; 14 to 19, mild depression;
20 to 28, moderate depression; 29 to 63, severe depression. Caregivers also completed the parent report Paediatric Quality of Life Inventory (PedsQL) (caregiver estimates of the quality of life of children) and PedsQL family impact module (caregiver estimates of quality of life of the family). Both are expressed as percentages, where 100% is no impact on quality of life, and 0% is severe impact on quality of life.

2.8.4 | Actigraphy

Participants wore Respironics Actiwatch 2 actigraphs (Philips Respironics, Bend, Oregon) on their non-dominant wrist continuously for 5 to 10 days, with data sampling across 15-seconds epochs.

Only participants for whom there were a minimum of 5 days continuous actigraphy recordings were included in the analysis: 13 at baseline (6 placebo, 7 genistein) and 12 at 12 months (7 placebo, 5 genistein).

Data was downloaded and analysed using El Temps (A. Diez-Noguera, University of Barcelona, Barcelona, Spain), following the procedures described previously,31,32. Parametric and non-parametric tests were applied to characterise activity rhythms. The parametric tests used were the $\chi^2$-periodogram,33 cosinor analysis and Rayleigh test,34 from which the following values were calculated: period of the rhythm, percentage of variance, cosinor, mesor, cosinor acrophase, cosinor amplitude and r vector length. In addition, using the mean waveform, the proportion of daytime activity in relation to the overall daily activity levels (%) was calculated, together with M10 activity levels, M10 duration, L5 activity levels and L5 duration (see Mumford et al for further details). The following non-parametric variables were also calculated31,32: interdaily stability (IS), intradaily variability (IV) and relative amplitude (RA).

2.8.5 | Measurement of cytokines in CSF and plasma

Concentrations of cytokines in in control and MPS III human plasma and CSF were evaluated using a commercially available Bio-Plex ProTM Human Cytokine 27-Plex Assay (Bio-Rad Laboratories Ltd., Watford, UK) measured with a Bio-Plex 200 system (Bio-Rad Laboratories Ltd.) powered by Luminex xMAP technology (Luminex, Austin, Texas), as described by Parker et al.35 Control samples were from healthy adults (plasma) or commercially available single donor CSF (Lee Biosolutions, Catalogue number 991-19-S).

2.9 | Statistical analysis

For CSF, plasma and urine HS, and urine GAG, analysis of covariance (ANCOVA) was used to compare genistein and placebo groups at 12 months, controlling for baseline value and for stratification variable (C vs non-C subtype). HS and GAG concentrations were log-transformed prior to analysis. Untransformed BSID-III and VABS-II DQ scores were analysed by ANCOVA, controlling for age at baseline, baseline DQ and MPS subtype. Untransformed BDI-II and PedsQL scores were analysed by ANCOVA controlling for baseline value and MPS subtype. Significance was set at 5%. Where log-transformed values were used, results are expressed as % difference between genistein and placebo after 12 months (adjusted for baseline). Analysis was performed using GraphPad Prism 7 software (La Jolla, California) and SPSS statistics v.24 (IBM, Armonk, New York).

Actigraphic data were analysed by ANOVA of general linear models using SPSS statistics v.22 (IBM, Armonk, New York). For all models, independent variables were treatment (placebo or genistein), experimental stage (baseline or 12 months) and interaction between these two variables.

Cytokine concentrations in genistein, placebo and control groups were analysed using one-way ANOVA, followed by Tukey's multiple comparisons test.

3 | RESULTS

3.1 | Baseline characteristics, safety and tolerability

Baseline characteristics are summarised in Table 1. Three of the four participants with MPS IIIC were randomised to the placebo group. Genistein was generally well tolerated by participants, with only one adverse event considered to be clearly related to genistein (Table S1). This was the development of Tanner stage II breast tissue in three male participants (aged 4.3, 7.9 and 7.6 years) that was not present at baseline. One participant who was initially allocated to the placebo group withdrew from the study shortly after commencing the open-label phase, due to reported decreasing responsiveness. Though no other patient reported similar symptoms, a relationship with genistein could not be completely excluded. Two male participants developed elevated testosterone levels during the study, one of whom had elevated gonadotropin levels and evidence of progression into puberty at 9.5 years of age. One female participant developed biochemical and clinical evidence of progression into puberty at 9.1 years of age. All participants were found incidentally to have
| Subject number | Sex | Age at baseline (m) | MPS III subtype | Gene | Allele 1 | Allele 2 | Baseline CSF HS (ng/mL) | Baseline cognitiveAgeEqS (m) baseline/1y/2y | BSID-III (cognitive DQ) baseline/1y/2y | BSID-III (composite DQ) baseline/1y/2y | VABS-II AgeEqS (m) baseline/1y/2y | VABS-II (composite DQ) baseline/1y/2y | Allocation |
|----------------|-----|---------------------|----------------|------|----------|----------|------------------------|-----------------------------------|--------------------------------|----------------------------------|--------------------------------|--------------------------------|------------|
| 1              | M   | 190                 | A              | SGSH | c.892T>C (p. (Ser298Pro)) | c.1027dupC | 1169 | 25/22/25 | 13/11/11 | 18/23/26 | 10/12/12 | Genistein |
| 2              | F   | 101                 | A              | SGSH | c.1298G>A (p. (Arg433Gln)) | Second variant not identified | 1791 | 14/8/NA | 14/7/NA | 13/10/NA | 13/9/NA | Genistein |
| 3              | M   | 74                  | A              | SGSH | c.1139A>G (p. (Gln390Arg)) | c.1295_1303del | 1460 | 8/8/7 | 11/9/7 | 10/10/6 | 13/12/6 | Genistein |
| 4              | M   | 80                  | B              | NAGLU | c.889C>T (p. (Arg297Ter)) | c.2020C>T (p. (Arg674Cys)) | 2248 | 8/9/16 | 10/10/15 | 19/15/16 | 23/17/15 | Genistein |
| 5              | M   | 86                  | B              | NAGLU | c.531+5G>C | c.2116C>T (p. (Gln706Ter)) | 1741 | 8/6/8 | 9/6/7 | 12/14/7 | 14/15/7 | Genistein |
| 6              | M   | 61                  | B              | NAGLU | c.291T>G (p. (Cys97Trp)) | c.291T>G (p. (Cys97Trp)) | 1269 | 11/NA/17 | 18/NA/20 | 18/NA/12 | 29/NA/13 | Genistein |
| 7              | M   | 89                  | A              | SGSH | c.571G>A (p. (Gly191Arg)) | c.571G>A (p. (Gly191Arg)) | 1481 | 10/8/7 | 11/8/6 | 22/21/15 | 25/20/13 | Genistein |
| 8              | F   | 66                  | A              | SGSH | c.697C>T (p. (Arg233Ter)) | c.1027dupC | 1448 | 10/10/8 | 15/12/9 | 16/12/11 | 25/15/12 | Genistein |
| 9              | F   | 97                  | C              | HGSNAT | c.947G>T (p. (Trp316Ter)) | c.1464+1 G>A | 2079 | 42/42/42 | 44/38/35 | 57/55/48 | 59/50/40 | Genistein |
| 10             | M   | 54                  | A              | SGSH | c.197C>G (p. (Ser66Trp)) | c.197C>G (p. (Ser66Trp)) | 1606 | 12/10/NA | 22/15/NA | 11/10/NA | 20/15/NA | Placebo |
| 11             | M   | 38                  | A              | SGSH | c.675C>G (p. (Phe224Leu)) | c.877C>T (p. (Pro293Ser)) | 1455 | 8/6/4.33 | 21/12/7 | 12/13/10 | 33/27/15 | Placebo |
| 12             | F   | 43                  | A              | SGSH | c.734G>A (p. (Arg245His)) | c.734G>A (p. (Arg245His)) | 2102 | 34/25/12 | 79/45/18 | 43/32/14 | 100/59/21 | Placebo |
| 13             | M   | 71                  | A              | SGSH | c.1430A>C (p. (Asp477Ala)) | c.1430A>C (p. (Asp477Ala)) | 1268 | 8/11/17 | 11/13/18 | 20/17/18 | 28/21/19 | Placebo |
| 14             | F   | 116                 | A              | SGSH | c.220C>T (p. (Arg74Cys)) | c.1080delC | 1292 | 7/4/3.7 | 6/3/5 | 9/6/6 | 7/5/4 | Placebo |
| 15             | F   | 182                 | A              | SGSH | c.548G>A (p. (Cys183Tyr)) | c.571G>A (p. (Gly191Arg)) | 1737 | 42°/NA/NA | 23°/NA/NA | 43/50/51 | 24/26/24 | Placebo |
| 16             | F   | 173                 | B              | NAGLU | c.2185_2187delAAG | c.2185_2187delAAG | 1670 | 8/7/5 | 5/4/3 | 10/12/8 | 6/6/4 | Placebo |
| 17             | F   | 178                 | C              | HGSNAT | No result available | No result available | 1222 | 42/38/27 | 24/20/13 | 37/34/31 | 21/18/15 | Placebo |
highly elevated sex hormone binding globulin (SHBG) at baseline (range 153-653 nmol/L). Six participants had elevated alanine transaminase (ALT) at baseline which did not deteriorate during the study (maximum 1.2-2.1 x upper limit of normal, ULN). A total of four participants across both genistein and placebo groups developed elevated ALT during the course of the study (maximum 1.2-2.3 x ULN). Five participants across both genistein and placebo groups developed elevated thyroid stimulating hormone during the study, one of whom had previously been treated for hypothyroidism.

3.2 | Treatment with 160 mg/kg/day genistein aglycone led to plasma and CSF genistein concentrations consistent with previous clinical and pre-clinical studies

Genistein aglycone at a dose of 160 mg/kg/day was delivered as two oral doses every 24 hours (0 hour,12 hours, 24 hours, 36 hours). The pharmacokinetic profile was assessed in all participants over a 12-hour period following their first dose and third doses of genistein (Figure 2A). Genistein is rapidly glucuronidated, or otherwise modified in humans, which significantly reduces activity. Thus, the peak plasma concentration of free and fully active genistein (genistein aglycone) in participants receiving genistein was measured as 339 ng/mL at 4 hours after the first dose and 508 ng/mL at 2 hours after the third dose. Plasma genistein concentrations were also measured at 12 months and 24 months (Figure 2B): mean plasma free genistein (in genistein group participants) was 84 ng/mL at 12 months (95% CI 27-142 ng/mL), and at 36 ng/mL at 24 months (95% CI 12-60 ng/mL). In participants originally on placebo, mean plasma free genistein concentrations were 87 ng/mL at 24 months (ie, after 12 months of genistein), 95% CI 22-153 ng/mL). Plasma free genistein concentrations were also measured in samples from previous clinical studies of genistein (Figure 2C). A dose of 10 mg/kg/day soy-derived genistein from the 30 patients on the Dutch trial was associated with mean plasma free genistein concentration of 13 ng/mL (95% CI 5.9-20.1 ng/mL), whereas a dose of 100 mg/kg/day from the US trial was associated with mean plasma free genistein of 90 ng/mL (~12.3-193 ng/mL). The plasma concentrations of genistein observed in the GENiSIS2013 trial are comparable to previous studies. Mean CSF concentrations of free genistein were 4.9 ng/mL in the genistein group at 12 months (95% CI 2.0-7.8 ng/mL); 4.9 ng/mL at 24 months (95% CI 2.7-7.2 ng/mL), and in participants initially in the placebo group, mean CSF free genistein after 12 months of genistein was 2.0 ng/mL
(95% CI 0.6-3.4 ng/mL) (Figure 2D). CSF concentrations were approximately 2% to 13% of plasma concentrations, consistent with pre-clinical studies.37

3.3 Treatment with 160 mg/kg/day genistein aglycone was not associated with significant reductions in CSF HS, plasma HS or urine HS, but there was a significant reduction in urine GAG

Patients were randomised to placebo or genistein for 12 months, following which all patients received genistein (Figure 3A). From baseline to 12 months, CSF HS decreased in the genistein group but increased in the placebo group. However, CSF HS increased in both groups from 12 months to 24 months, when all participants were receiving genistein (Figure 3B). The difference in CSF HS between genistein and placebo groups after 12 months was not statistically significant in the ANCOVA analysis (estimated difference: 5.5% lower in genistein group, 95% CI: 14.7% lower to 4.7% higher, P = .260) (Figure 3C). Thus, the study did not meet its primary endpoint of a reduction in CSF HS from baseline to 12 months. Mean concentrations of plasma HS, urine HS and urine GAG decreased in both groups over time (Figure 3D,E,F). There was a statistically significant change in plasma HS concentration from baseline to 12 months (P = .021) and from baseline to 24 months in the genistein group (P = .027), but not in the placebo group (Table S2). However, this was not statistically significant in the ANCOVA analysis (estimated difference at 12 months: 10.4% lower in genistein group, 95% CI: 23.4% lower to 4.9% higher, P = .159). Similarly, the estimated difference in urine HS between genistein and placebo after 12 months of treatment was not statistically significant (5.1% lower in genistein group, 95% CI: 30.8% lower to 30.0% higher, P = .73). The only biochemical outcome measure for which there was a significant difference between groups in ANCOVA analysis was urinary GAG at 12 months (32.1% lower in the genistein, 95% CI: 53.8% lower to 0.1% lower, P = .0495). However, urinary GAG concentrations

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**Figure 2** Pharmacokinetic profile of genistein and plasma/CSF genistein concentrations. Data shown are means with 95% CI. A, Pharmacokinetic profile of genistein after dosing at 0 hour, 12 hours, 24 hours, 36 hours. Blue arrows denote timing of genistein doses. B, Genistein concentration in plasma in participants in this study at baseline, 12 months and 24 months. C, Genistein concentrations in plasma in participants from de Ruijter et al25 and Kim et al26 studies. D, Genistein concentrations in CSF in participants in this study at baseline, 12 months and 24 months.
remained well above the normal range in both groups (Figure 3F).

3.4 No differences in neuropsychological or quality of life measures were observed between placebo and genistein groups

The developmental trajectory of MPS IIIA, IIIB and IIIC individuals in both genistein and placebo groups closely followed the curves of published natural history data for MPS IIIA throughout the 24 months of the study. This included a small subset of individuals from both MPS IIIC and non-C subtypes who followed more attenuated courses (Figure 4A,B). The decline in DQ over the first 12 months was greater in the placebo group (mean decline of 9.6 points) than in the genistein group (mean decline of 3.2 points) (Table S3). However, many individuals in the genistein group had low baseline DQ, and clustered near the “floor” of the test, whereas there were several younger individuals in the placebo group with higher baseline DQ. Age at baseline was therefore controlled for in the analysis, and the difference between groups at 12 months was small and not statistically significant (1.4 points higher in the genistein group, 95% CI 1.7 points lower to 4.6 points higher, \( P = .347 \)).

Results for adaptive behaviour followed a similar pattern (Figure 4C,D). There was no statistically significant difference between genistein and placebo groups at 12 months (mean difference 0.3 points lower in the genistein group, 95% CI 4.1 points lower to 4.8 points higher, \( P = .868 \) (Table S2)).

Caregiver mood was assessed using the BDI-II. At baseline, mean BDI-II scores in the genistein group ranged from minimal to moderate depression and from minimal to severe depression in the placebo group (Table S3). The BDI-II score for a parent of one participant in the placebo group increased from 20 at baseline to 46 at 12 months, prompting a referral to primary care services for further assessment. Mean changes in scores during the course of the study were small (Figure 4E). The difference between groups at 12 months, adjusted for baseline, was small and not statistically significant (3.9 points lower in the genistein group, 95% CI: 13.3 points lower to 5.5 points higher) (Table S3). Parents Paediatric Quality of Life Questionnaires (PedsQL) and PedsQL Family Impact module scores at baseline indicated significant impact on the parents'...
FIGURE 4  Neuropsychological outcome measures and quality of life measures. A, Bayley age equivalent scores by chronological age, with MPS IIIA natural history. B, Bayley DQ by chronological age, with MPS IIIA natural history. C, Vineland age equivalent scores by chronological age. D, Vineland DQ by chronological age. E, Beck Depression Inventory scores (0–13 = minimal depression; 14–19 mild depression; 20–28 = moderate depression; 29–63 = severe depression). F, Parents PedsQL scores, given as percentages, where 100% = no impact on quality of life of child and 0% = severe impact on quality of life of child. G, PedsQL Family Impact Inventory, given as percentages, where 100% = no impact on quality of life of family and 0% = severe impact on quality of life of family. For panels (A-D), data shown are individual participants. For panels (E-G), data shown are means with 95% confidence intervals. DQ = [age equivalent score/chronological age] × 100. Circles with dashed connecting line = genistein; squares with solid connecting line = placebo; squares with dotted connecting line = placebo group changed to genistein during extension phase; open circles = MPS IIIC participants randomised to genistein; open squares = MPS IIIC participants randomised to placebo and changed to genistein during extension phase; triangles = Stanford-Binet scores for one MPS IIIA participant who scored above the limits of Bayley scales; grey lines without symbols = MPS IIIA natural history data from Shapiro et al.38; grey dashed lines without symbols = MPS IIIA “slow progressors” from Shapiro et al.38 Solid lines in panels (A) and (C) represent trajectory of normal development. Bayley, Bayley Scales of Infant and Toddler Development, Third Edition; DQ, developmental quotient; Vineland, Vineland Adaptive Behaviour Scales, Second Edition.

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perceived quality of life of their child and the parents’ reported quality of life of the family (Table S3). From baseline to 12 months, the Parents PedsQL scores improved in the genistein group and deteriorated in the placebo group (Figure 4F), and approached statistical significance (10.0 percentage points higher in the genistein group, 95% CI: 0.6 percentage points lower to 20.6 percentage points higher, \( P = .061 \)) (Table S3). However, scores deteriorated in both groups from 12 months to 24 months (Figure 4F). Family Impact scores improved by a greater degree in the genistein group from baseline to 12 months, though this was not statistically significant, and again, scores deteriorated in both groups from 12 months to 24 months.

3.5 No statistically significant changes in actigraphic outcome measures were observed between genistein and placebo groups

Several actigraphic variables differed between treatment groups, though this was at both baseline and 12-month time points. Across both timepoints, the genistein group showed higher levels of daytime activity (94.3 ± 2.4%) than the placebo group (91.2 ± 3.5%) (\( P < .05 \), data not shown). Similarly, across both timepoints, cosinor amplitude, which indicates strength of the rhythm, was significantly higher in the genistein group (4394 ± 1904) compared to the placebo group (3232 ± 1630) (\( P < .05 \)). Intradaily variability (IV), which gives an indication of the level of fragmentation of the rhythm, was also significantly higher in the genistein group (0.70 ± 0.18) than in the placebo group (0.53 ± 0.17) (\( P < .05 \)). However, there was no statistically significant interaction between treatment group and timepoint, suggesting that there was no independent treatment effect of genistein.

The period of the activity rhythm was shorter at baseline (23.8 ± 0.3 hour) compared to the 12-month timepoint (24.1 ± 0.2 hour), but this was across both treatment groups (\( P < .05 \)). No other significant interaction between treatment group and timepoint was found.

3.6 Cytokines

Significant elevations in certain cytokines (including IL-1β, IL1RA, MCP-1, MIP-1α and MIP-1β) were seen in participants over unaffected individuals, but there were no significant differences in the change from baseline to 12 months for any cytokine as a result of genistein treatment (data not shown). Patients in the genistein group generally had higher baseline cytokine levels than those in the placebo group which confounded analysis.

4 DISCUSSION

This is the first double-blinded, placebo-controlled clinical trial of high dose genistein aglycone in patients with MPSIII that was designed to specifically address whether high dose genistein improves neurological disease symptoms. We show here that although we could demonstrate that significant amounts of genistein were present in the blood circulation as a result of dosing, and despite being able to demonstrate a small but significant reduction in urinary GAGs, we did not demonstrate any effect on neurocognition, psychological well-being of individuals or families, or other clinical symptoms including inflammatory cytokine levels or actigraphy.

Initial open label clinical studies of genistein delivered as SOYFEM (a standardised soy isoflavone extract) in patients at doses of between 5 and 10 mg/kg/day genistein aglycone were inconclusive, with one delivering 5 mg/kg/day suggesting small improvements in 10 patients, whilst similar studies in Spain24 in 19 Sanfilippo patients, suggested no clinical effect. A previous placebo-controlled study using doses of 10 mg/kg/day Soyfem demonstrated a significant reduction of urinary GAGs, as we showed here, but they too were unable to show clinical improvements or stabilisation in neurocognitive tests. A later study in the US using very high doses of genistein aglycone 60 to 150 mg/kg/day26 was not set up to monitor clinical outcomes. As we demonstrate here, the patients from the de Ruijter study had an average circulating genistein level of 13 ng/mL, (Figure 2C) whereas with a dose 16-fold higher, we achieve between 339 and 508 ng/mL at cMax from our PK data (Figure 2A) and 36 to 87 ng/mL steady state levels in our long-term measurements (Figure 2B, D), suggesting that we should have seen a larger effect size on GAG or HS reduction. Sporadic measurements from patients on the US study receiving 60 mg/kg or 100 mg/kg genistein aglycone were consistent with this observation (Figure 2C).

CSF bioavailability of genistein aglycone was slightly lower than expected (between 2% and 13% of input), although not inconsistent with previous reports of 10% bioavailability in the brains of rats19 and our own data in mice (data not shown) of 8.5% brain bioavailability. It is possible that the genistein aglycone levels achieved in the brain in this study (2-5 ng/mL) remained too low to achieve a biochemical effect and this was borne out by a lack of significant changes in CSF HS or in neurocognition in the treated group. Some clues may be seen from a dose finding study in male MPSIIIB mice20 that show initial dose dependent decreases in liver GAGs between 0-15 mg/kg/day, and no further decreases between 15-160 mg/kg/day, suggesting that genistein
may have an L-shaped dose response curve on GAG reduction. In contrast, a long-term double-blinded study of high dose genistein aglycone demonstrated a consistent 37% reduction of HS in the brain of MPSIIIB mice, suggesting that bioavailability could be an issue. Nonetheless, we found plasma and brain bioavailability of the active unconjugated “free” form of genistein (genistein aglycone) to be very similar between mice and humans, although plasma levels of the less active conjugated genistein form were much higher in humans due to higher glucuronidation and sulphation rates of oestrogenic compounds such as genistein in humans. The data therefore suggested that there should have been sufficient bioavailability of genistein aglycone in both plasma and the CSF to achieve GAG reductions. The hypothesis that reduction of heparan sulphate in Sanfilippo could impact positively on disease status is still very much viable, and can be demonstrated in several preclinical models using siRNA, shRNA or genetic models designed to reduce HS synthesis. Genistein, however, does not produce a significant enough effect size to impact on pathology in humans.

Higher dosing of genistein would not be recommended, as the NOAEL for chronic dosing is under 500 mg/kg/day in rodents and 500 mg/kg/day in dogs. Indeed, Kingma and colleagues recently described adverse effects of genistein in mouse models of the related GAG storage disease MPS I, including decreased femur and body length and 60% of mice with scrotal hernia. In the study by Kim and colleagues of participants receiving 150 mg/kg/day genistein aglycone, two boys developed Tanner stage II breast development and two female participants developed menstrual irregularities during the course of the study. In comparison, three boys developed Tanner stage II breast development in this study. Similarly, we observed moderate elevations in alanine transaminase in four participants that did not exceed three times the upper limit of normal, as was described in three participants in the study by Kim and colleagues. In conclusion, we found that genistein is safe and well tolerated in MPSIII patients.

We compared our age equivalent cognitive scores to a natural history study of 20 MPSIIIA patients published by Shapiro and colleagues. Although we had a mixture of MPS IIIA, MPS IIIB and MPS IIIC patients on this trial, and despite some obvious differences in course of disease for some patients who had an attenuated phenotype, it was clear that there were no changes to overall rate of decline between placebo or genistein groups as measured for either age equivalent DQ or adaptive behaviour. We observed attenuated phenotypes in two patients with MPS IIIA (Table 1, subjects #1 and #15) and one patient with MPS IIIB (Table 1, subject #16). Subject #1 was compound heterozygous for p.(Ser298Pro), a variant known to be associated with slowly progressing MPS IIIA, and c.1027dupC, which has previously been observed in a homozygous state in rapidly progressing individuals. Subject #15 was compound heterozygous for p.(Gly191Arg), a variant which has previously observed in trans with other variants in individuals with rapidly progressing MPS IIIA, and a novel variant, p.(Cys183Tyr). Subject #16 was homozygous for a novel in-frame deletion in NAGLU. Overall baseline HS values did not appear to be different for different rates of progression or subtype suggesting that HS levels are not prognostic at baseline for severity of disease. A slower clinical course of disease has been well documented for MPS IIC, and our findings were consistent with this.

This study recruited participants from a broad age range, with the youngest participant being 38 months old at baseline. Participants under 2 years of age were not recruited as such children would potentially have been eligible for participation in the increasing number of gene therapy clinical trials that were becoming available during this period. However, the heterogeneity of the participants is a limitation of the study. In addition, many of the participants already had marked impairment on neuropsychological assessments at baseline, limiting the potential of the study to identify differences in cognitive outcomes. For this reason, a biochemical outcome measure (CSF HS) was chosen as the primary endpoint.

The difficulty of using CSF HS reductions as a clinical surrogate for brain HS or even a predictor of neurocognitive outcomes in MPSIII have previously been highlighted by us and others. Gene therapy studies in MPS IIIA and MPSIIIB have further brought this into context, where significant reductions in CSF HS have been seen without consequential changes in neurocognitive outcomes. Given the difficulties of achieving neurocognitive changes in this population with an enzyme replacement gene therapy, and the suggestion that reductions of 50% to 70% of brain HS may be needed to achieve this from our own preclinical gene therapy studies in MPS IIIA, MPS IIIB and MPS IIIIC, it seems unlikely that any substrate reduction therapy product could reduce GAG sufficiently to achieve improvements in neurocognition in MPS III. Miglustat is approved as a substrate reduction therapy for Gaucher disease, however, it has a dual function as a chaperone and has a much more specific action on its substrate than genistein. As such, any future SRTs should be more specifically targeted and may need to be able to reduce GAG by over 50% in the brain in order to stand a chance of clinical success.

Despite seeing small but significant reductions in the preclinical mouse study of high dose genistein in
MPSIIIB mice, we were unable to show any significant reductions in a panel of elevated cytokines in MPS III patients. These data further suggest that the effect size of genistein in humans was lower than expected and suggest that although the product has sufficient bioavailability, it is unable to reduce GAGs or reduce neuroinflammation sufficiently to mediate any detectable changes in patients. Preclinical data from our laboratory has suggested that behaviour in MPSIIIA can be influenced by reduction of inflammation in the absence of storage reduction. As genistein has multiple effects, including a small but significant reduction in brain inflammation in MPSIIIB mice, this, coupled with modest GAG reductions may also help to explain why it was ineffectual in patients.

In conclusion, our data strongly suggest that despite sufficient bioavailability of high dose genistein aglycone in patients receiving the drug for up to a 2-year period, there is no clinical benefit to be seen for neurological and neurocognitive signs and symptoms of MPS III. In addition, biochemical somatic benefits were marginal at best, and similar effects could be achieved with much lower doses of genistein in Soyfem form. As such, we do not recommend the use of genistein aglycone at any dose for the treatment of MPS III or other similar neurological diseases storing glycosaminoglycans.

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CONFLICT OF INTEREST
A.G. declares travel grant from Biomarin, unrelated to the submitted work; S.R. declares travel grants and speaker fees from Takeda, Vitaflno, unrelated to the submitted work; K.L. has no conflicts to declare; D.W. has no conflicts to declare; M.C. has no conflicts to declare; C.B. has no conflicts to declare; M.H. has no conflicts to declare; K.T. has no conflicts to declare; F.V. has no conflicts to declare; A.V. has no conflicts to declare; F.W. has no conflicts to declare; C.O. has no conflicts to declare. H.P. has no conflicts to declare; J.E.W. N/A; B.B. declares being an S.A.B member and shareholder of Orchard Therapeutics and the recipient of unrestricted clinical trial grants from Orchard Therapeutics and Avrobio -unrelated to the submitted work; S.J. declares being investigator, SAB member and stockholder of Orchard therapeutics, and investigator and consultant for Takeda (formally Shire HGT), unrelated to the submitted work.

AUTHOR CONTRIBUTIONS
Arunabha Ghosh, Stewart Rust, Kia Langford-Smith, Catherine Breen, Andy Vail, Claire O’Leary, J. Ed Wraith, Brian W. Bigger, Simon A. Jones: Conception and design; Arunabha Ghosh, Stewart Rust, Daniel Weisberg, Maria Canal, Catherine Breen, Michelle Hepburn, Karen Tylee, Frédéric M. Vaz, Andy Vail, Frits Wijburg, Helen Parker, Brian W. Bigger, Simon A. Jones: Acquisition and analysis of data; Arunabha Ghosh, Stewart Rust, Daniel Weisberg, Maria Canal, Brian W. Bigger, Simon A. Jones: Drafting of manuscript and figures.

ETHICS STATEMENT
Ethical approval was given by the Office for Research Ethics Committees, Northern Ireland (ORECNI) (REC 14/NI/0006). Informed consent was obtained for all participants and the study was conducted in accordance with the principles set out in the WMA Declaration of Helsinki.

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SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of this article.

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