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High Sustained Antibody Titers in Patients with Classic Infantile Pompe Disease Following Immunomodulation at Start of Enzyme Replacement Therapy

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Objective To evaluate whether immunomodulation at start of enzyme replacement therapy induces immune tolerance to recombinant human acid alpha-glucosidase (rhGAA) in patients with classic infantile Pompe disease.

Study design Three patients (1 cross reactive immunologic material negative, 2 cross reactive immunologic material positive) were treated with 4 weekly doses of rituximab, weekly methotrexate, and monthly intravenous immunoglobulin and enzyme replacement therapy at 40 mg/kg/week. Antibody titers were measured using enzyme-linked immunosorbent assay. Neutralizing effects on rhGAA activity and cellular uptake were determined and combined with pharmacokinetic analysis. Clinical efficacy was evaluated by (ventilator-free) survival, reduction in left ventricular mass index, and improvement of motor function.

Results Immunomodulation induced B cell depletion that was accompanied by absence of antibody formation in all 3 patients. Upon cessation of rituximab treatment, all 3 patients showed B cell recovery, which was accompanied by formation of very high sustained antibody titers in 2 patients. Neutralizing effects on infused rhGAA were low to mild/moderate. All patients were alive at study end, learned to walk, and showed (near) normalization of left ventricular mass index.

Conclusions Immunomodulation as recommended in the literature prevented formation of rhGAA antibodies only during B cell depletion but failed to induce immune tolerance in 2 out of 3 patients. *(J Pediatr 2018;195:236-43).*

Pompe disease (Glycogen Storage Disease type II, OMIM: #232300) is an autosomal recessive lysosomal storage disorder caused by deficiency of the enzyme acid-α-glucosidase (GAA), and results in lysosomal glycogen accumulation with prominent pathology in cardiac and skeletal muscle cells.[1] Pompe disease presents as a spectrum of clinical phenotypes. The classic infantile form is the most severe, with residual alpha-glucosidase activity in cultured fibroblasts of less than 1%. About two-thirds of the patients with classic infantile Pompe disease produce inactive GAA protein, and they are, therefore, called cross-reactive immunologic material (CRIM) positive; one-third does not produce any detectable GAA protein, and these patients are called CRIM negative. Without treatment, patients with classic infantile Pompe disease die within the first year of life because of cardiorespiratory failure.[2-4]

Enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA, alglucosidase alfa, Myozyme) has led to significant improvement of the prospects of patients with classic infantile Pompe disease, but the clinical response to ERT is heterogeneous: nearly one-half of patients do not survive ventilator-free beyond the age of 3 years.[5-9] One of the factors that may reduce the efficacy of ERT is the formation of antibodies to rhGAA that neutralize its activity or prevent its cellular uptake.[10-13] Antibodies potentially pose a threat to survival because classic infantile patients are strongly dependent on ERT.[14-16] In general, a CRIM negative status is associated with high probability to form anti-rhGAA antibodies and a poor clinical outcome.[11,12] However, many CRIM positive classic infantile and some childhood/adult onset patients with Pompe (which are by definition CRIM

AIMS	Alberta Infant Motor Scale	IARs	Infusion-associated reactions
ALT	Alanine transaminase	IVIG	Intravenous immunoglobulin
AST	Aspartate transaminase	LVMI	Left-ventricular mass index
CRIM	Cross-reactive immunologic material	MTX	Methotrexate
ELISA	Enzyme-linked immunosorbent assay	rhGAA	Recombinant human GAA
ERT	Enzyme replacement therapy	RTX	Rituximab
GAA	Acid-α-glucosidase		

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positive), also develop high anti-rhGAA antibody titers. The likelihood to form high and sustained antibody titers is difficult to predict.[11,12,17,18]

In 2012, prevention of antibody formation was shown by immunomodulation/immune tolerance induction at the start of ERT.[19,20] The protocol consisted of a 4-week treatment with rituximab (RTX), and additional treatment with methotrexate (MTX) and intravenous immunoglobulin (IVIG). The rationale was to prevent formation of B memory cells at the start of ERT by eliminating B cells with RTX during the initial 4 weeks of ERT. MTX was used as additional immunomodulatory agent, and IVIG supplementation was applied to prevent opportunistic infections.

We implemented this primary immunomodulation protocol[19,20] in our patients, and monitored: formation of anti-rhGAA antibodies, depletion and repopulation of B cells, effects of antibodies on GAA enzyme activity and cellular uptake, and clinical outcome. Furthermore, we determined the effects of antibodies during infusion with ERT.

Methods

Study protocols were approved by the Institutional Review Board, and written informed consent was obtained from the parents. Standardized assessments were performed at baseline, on a monthly basis during the first 3 months, and every 3 months thereafter as described by van den Hout et al.[6] Immunomodulation consisted of intravenous RTX 375 mg/m^2/dose, weekly for 4 weeks followed by intravenous MTX 1 mg/kg/dose based on Messinger et al and Mendelsohn et al.[19,20] The first dose of RTX was administered the day before the first dose of alglucosidase alfa dosed at 40 mg/kg/week. MTX was given directly after RTX 1 day before the infusion during the first 4 weeks and from week 5 onward weekly MTX was given directly after ERT infusion. IVIG at 400 mg/kg was given monthly starting at week 4 until B cell levels and IgG levels had reached normal values for age. Antibiotic prophylaxis was given to prevent (respiratory) infections. Regular blood analysis consisted of assessing number of neutrophils, number of B and T cells, aspartate transaminase (AST), alanine transaminase (ALT), creatine kinase, and gamma globulin (IgG, IgM, IgA) levels. MTX was temporarily discontinued when neutrophil counts were below 0.50 × 10^9 g/L, AST/ALT levels became more elevated than expected based on the disease course, or when patients had a temperature >38.5°C

Patients treated for at least 24 months with ERT and immunomodulation were included. Immunomodulation was performed in patients, regardless of their CRIM status, who were older than 2 months of age at the start of ERT, as these might have the highest risk to develop high anti-rhGAA antibody titers.[12] Here we report on the first 3 patients from our center who received immunomodulation. Classic infantile Pompe disease was defined as symptoms of muscle weakness within 6 months after birth, the presence of hypertrophic cardiomyopathy, confirmed deficiency of endogenous α-glucosidase in fibroblasts of <1% of the normal mean, and mutation analysis.

Clinical outcome was evaluated by (ventilator-free) survival. Cardiac dimensions were measured by conventional echocardiography according to the recommendations of the American Society of Echocardiography; left ventricular mass index (LVMI) was calculated by the Devereux formula and indexed by body surface area. An LVMI of >+2SD of age-related peers was considered abnormal.[21,22] Motor function was assessed by Alberta Infant Motor Scale (AIMS), Bayley Scales of Infant Development II, and by recording motor milestones.[23,24] Infusion-associated reactions (IARs) were noted when they occurred.

Anti-rhGAA titers were assessed at baseline, 4, 8, and 12 weeks after start of ERT and thereafter every 3 months. Standardized antibody analysis was performed by an enzyme-linked immunosorbent assay (ELISA) as described.[12,17,18] Antibody titers were measured in a 5-fold serial dilution to determine the antibody titer range. When a positive titer was found, a 2-fold serial dilution was used to obtain a more precise titer. The highest titer measured was used. Samples were measured in duplicate per assay, and assays were performed at least twice. The background of the ELISA method was determined to be 1:250 by omitting the coating of the plates with rhGAA as described previously.[17]

Blood was drawn before start of infusion with alglucosidase alfa, at several preset time points during the infusion, and after completion. Plasma or serum was prepared and stored at −80°C until use. The effect of antibodies during infusion of ERT was determined by capturing antibody-rhGAA complexes using protein A or G sepharose, followed by analysis of the supernatant on rhGAA enzyme activity by 4-MU assay as described[18] and rhGAA protein content by mass spectrometry as described.[25] Results obtained using protein A or protein G sepharose were similar.

Neutralizing effects of antibodies were measured as described.[12,17] In short, to fibroblasts from a patient completely deficient for endogenous GAA activity, 20 μL of the patient's serum was added along with 200 nmol rhGAA for a total volume of 200 μL Ham's-F10+ medium containing 3 mM PIPES and antibiotics. Enzyme activity was measured in medium and in cell lysate and compared with the activity in a fetal calf serum sample (set at 100%).

For in vitro analysis, 10 nmol rhGAA in 10 μL Phosphate buffered saline (PBS) was incubated with various dilutions of patient's sera in the presence of 40 μL protein A/sepharose for 1 hour at room temperature. The mixture was centrifuged for 2 minutes at 12 000 RPM in an Eppendorf centrifuge. Enzyme activity in the supernatant was measured and compared with the results obtained with sera from control individuals.

Results

Patient characteristics are given in **Table I**. Patient 1 was homozygous for c.525delT, which leads to undetectable GAA messenger RNA or protein expression. This patient was, therefore, CRIM negative.[26] Patient 2 was homozygous for c.1551+1G>A. Immunoblot analysis showed that this *GAA* variant led to GAA protein production, and the patient was, therefore, CRIM

Table I. Patient characteristics

Characteristics	Patient 1	Patient 2	Patient 3
At baseline			
Sex	Male	Female	Male
Age at diagnosis (mo)	5.7	4.0	2.9
Age at start of ERT (mo)	5.8	4.3	3.1
GAA variants	c.525delT c.525delT	c.1551+1G>A c.1551+1G>A	c.525delT c.2481+102_2646+31del
CRIM status*	Negative	Positive	Positive
Enzyme activity in fibroblasts/leucocytes†	0.3/1.6	0.1/1.6	0.4/1.3
LVMI at baseline/z score‡	265/26.1	753/87.8	160/12.8
AIMS at baseline (percentile score)	4 ($p < 5$)	3 ($p < 5$)	3 ($p < 5$)
At study end			
Age (mo)	34	28.2	27.0
LVMI/z score	65/0.7	87/3.5	72/1.6
Maximum motor milestone (age achieved in mo)	Walking (21.3)	Walking (18.0)	Walking (17.3)
Number of IARs (number of severe IARs)	15 (3)	0	0
Peak antibody titer (mo of ERT treatment)	800 000 (13)	6250 (14)	200 000 (14)
B cell recovery (time in mo)§	6.7	6.7	7.7
B cell level (age at study end)§	1.25*10E9 (30.2)	0.75*10E9 (25.4)	0.67*10E9 (22.4)
IgG level (age at study end)¶	4.6 (30.2)	6.5 (25.4)	3.3 (22.4)

*CRIM status in patient 1 and 3 was based on known effects of *GAA* variants. In patient 2, CRIM status was assessed by immunoblot analysis of fibroblasts (**Figure 1**).
†Enzyme activity in nmol/h/mg (fibroblasts: 4-MU substrate; leucocytes: 4-MU substrate + acarbose). Normal range fibroblasts: 45-180 nmol/hr/mg. Normal range leucocytes: 6.7-27 nmol/hr/mg.
‡LVMI in g/m^2.
§Time to B cell recovery in months after the last dose of RTX. B cell normal range: 0.2-2.1*10E9.
¶IgG normal range: 3.5-10.0 g/L.

positive (**Figure 1**; available at www.jpeds.com). This is in agreement with splicing analysis of this variant, which indicated that it causes a perfect skip of exon 10 in which the reading frame remains intact.[27] Patient 3 contained the c.525delT and the c.2481+102_2646+31del (delex18) *GAA* variants, the latter of which expresses a truncated GAA protein, and, therefore, this patient was also CRIM positive.[28]

Patients started ERT at a relatively late age (5.8, 4.3, and 3.1 months). At start of ERT, all 3 patients were able to move and lift arms and legs from the surface, showed poor head balance, and none were able to roll over. AIMS scores were clearly below the fifth percentile in all 3 patients. Two patients required oxygen by nasal prong, the other was monitored at night because of superficial breathing. All required nasogastric tube feeding and had a hypertrophic cardiomyopathy (**Table I**). The LVMI value was the highest in patient 2, who showed additional dilated cardiomyopathy (**Table I**). To exclude other possible causes of cardiac disease in this patient, exome sequence analysis of a panel of 49 genes known to be involved in hypertrophic cardiomyopathy was performed (**Table II**; available at www.jpeds.com). No other pathogenic variant was found.

The number of B cells decreased to undetectable levels after start of RTX treatment, and normalized in all patients after RTX was stopped. Time to B cell recovery was 6.7 months (patient 1); 6.7 months (patient 2); and 7.7 months (patient 3) (**Figure 2**). T cells remained within normal values throughout the study in all 3 patients (data not shown). During B cell depletion, all patients received IVIG; this was continued after B cell recovery until gamma globulin levels remained within the normal range for age (>3.5 g/L). IVIG was stopped in patient 1 at 18.7 months after start of immunomodulation and in patient 2 at 12.6 months. At study end, patient 3 still needed IVIG with 2- to 3-month intervals. These results indicate that all 3 patients responded to the immunomodulatory treatment with a rapid and transient B cell depletion.

The immunomodulation regimen was well tolerated. Patients 1 and 3 showed a mild skin reaction during the first dose of RTX. In patient 2, the third RTX infusion was skipped because of a concomitant bacterial infection at that time. In patient 1, MTX treatment was temporarily suspended at 11 months after start of immunomodulation for 4 weeks because of unanticipated increase of AST and ALT levels. MTX was restarted at a lower dose of 0.5 mg/kg/week. In total, 9 of the weekly MTX administrations were skipped over the course of 24 months (**Figure 2**). In patient 3, 1 dose of MTX was skipped because of low neutrophil count 21 months after start. IARs during alglucosidase alfa infusions occurred only in patient 1. Fifteen mild IARs and 3 severe IARs were observed (as defined in **Table III**; available at www.jpeds.com). The age at the first IAR was 6.7 months (infusion 5; first asterisk in **Figure 2**, A). Antihistamines were administered before the next infusions and the infusion rate was adapted. Antihistamines were stopped at age 8.5 months (infusion 14). Infusion rates were gradually adapted to higher rates, starting at the age of 14 months (8.2 months of ERT). This process was still ongoing at the age of 20.7 months (14.8 months of ERT) when he experienced a new IAR (second asterisk in **Figure 2**). At that time, infusion rates were slowed down again and antihistamines and corticosteroids were started. With this regimen, IARs remained variably present until the end of the study period.

Antibody titers as measured with ELISA are shown in **Figure 2**. In patient 1, anti-rhGAA antibodies were first detected at 4.5 months of ERT (titer 1:1250) and continued to increase to a maximum titer of 1:800 000 at 13 months of ERT. In patient 2, anti-rhGAA antibodies were undetectable during the first 9 months of ERT. At 12 months of ERT, the antibody

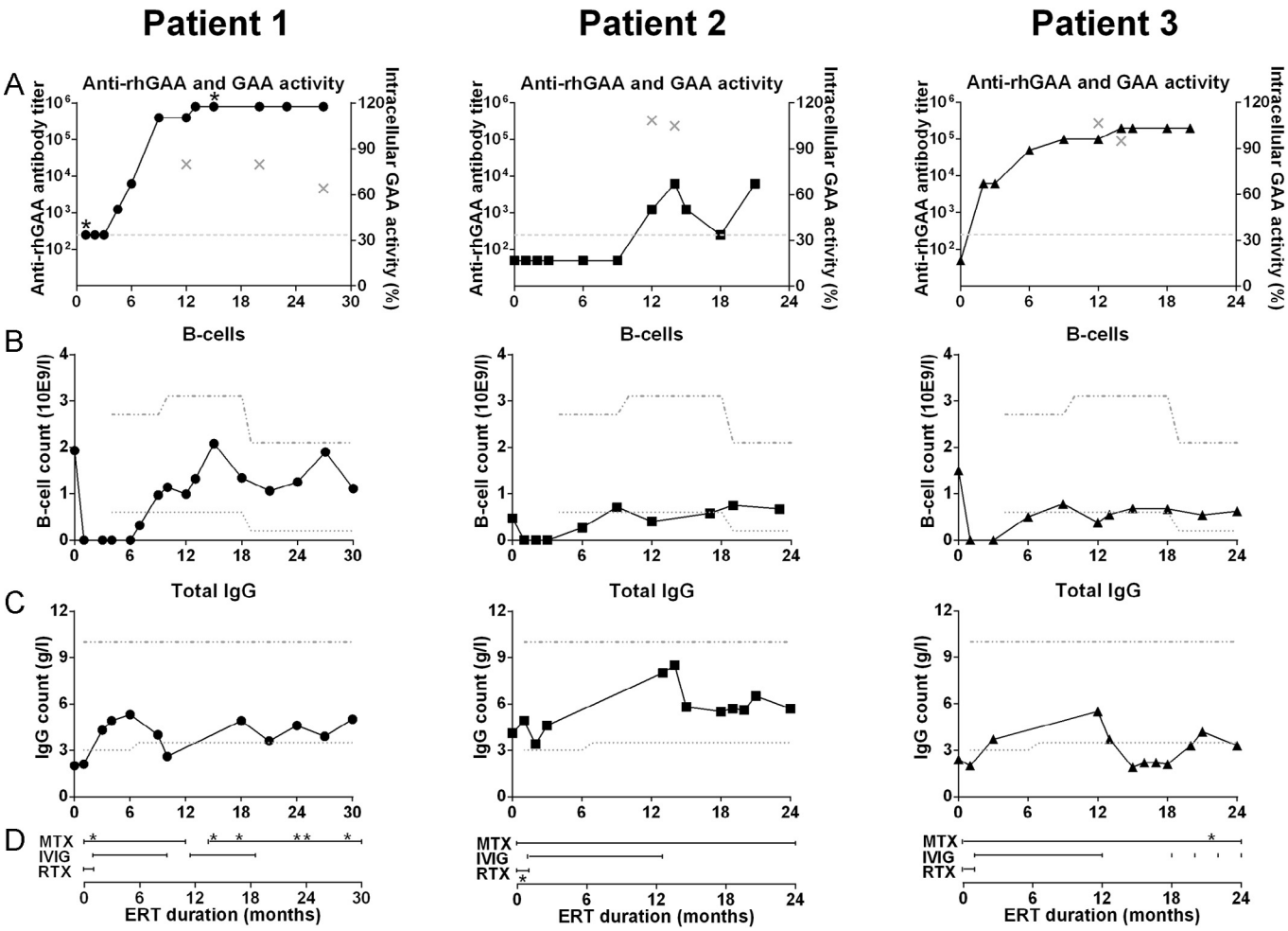

Figure 2. Immunologic responses during immunomodulation. Each column represents a single patient. **A,** Anti-rhGAA antibody titers (*black symbols, left y-ax*is) and neutralizing effects of anti-rhGAA antibodies (*gray crosses on right y-a*xis). The intracellular rhGAA activity is determined by incubation of fibroblasts with patient serum plus rhGAA (alglucosidase alfa) and is expressed as percentage of control serum (fetal calf serum). Black asterisk indicates the first and subsequent IAR in patient 1. Dashed gray lines represent background levels of the ELISA. **B,** B cells levels; dashed gray lines indicate normal values in serum. **C,** Total IgG levels; dashed gray lines indicate normal values in serum. **D,** Immunomodulation schedule per patient, each line represents treatment duration. In patient 1 and 3, the black asterisk indicates missed MTX injection(s); in patient 2 black asterisk indicates missed RTX infusion.

titer was 1:1250 and increased to an intermediate maximum titer of 1:6250 at 14 and 21 months of ERT. In patient 3, anti-rhGAA antibodies were detected at 2 months of ERT (1:6250), and increased to a very high maximum titer of 1:200 000 at 14 months of ERT. The start of antibody formation coincided with B cell recovery. Titers remained high in patients 1 and 3, and fluctuated between intermediate and background levels in patient 2 during follow up (**Figure 2**). These results indicated that the immunomodulation procedure induced a temporarily suppression of antibody formation during B cell ablation, while it failed to induce immune tolerance.

To test for neutralizing effects of antibodies, GAA-deficient fibroblasts were incubated with alglucosidase alpha plus patients' serum, followed by enzyme activity measurements in medium and cell lysates (**Figure 2**). The latter was taken as the sum of neutralizing effects on cellular uptake and inhibition of enzymatic activity. A reference patient that we reported previously,[18] served as a positive control for detection of neutralizing antibodies; this patient showed an enzyme activity of 29.7% in the cell lysate (data not shown). No neutralizing effects of antibodies in the medium were found (enzyme activities in all case were >92% of control; data not shown). In patients 1, 2, and 3, enzyme activities in cell lysates were 79.8%, 105%, and 94.8% as percentage of control at 20, 14, and 14 months of ERT, respectively (**Figure 2**; gray crosses, right axis), indicating moderate (patient 1) to no (patients 2 and 3) neutralizing effects. However, at 27 months of ERT, neutralizing effects of antibodies in patient 1 were more pronounced and indicated 60% residual enzyme activity in cell lysates.

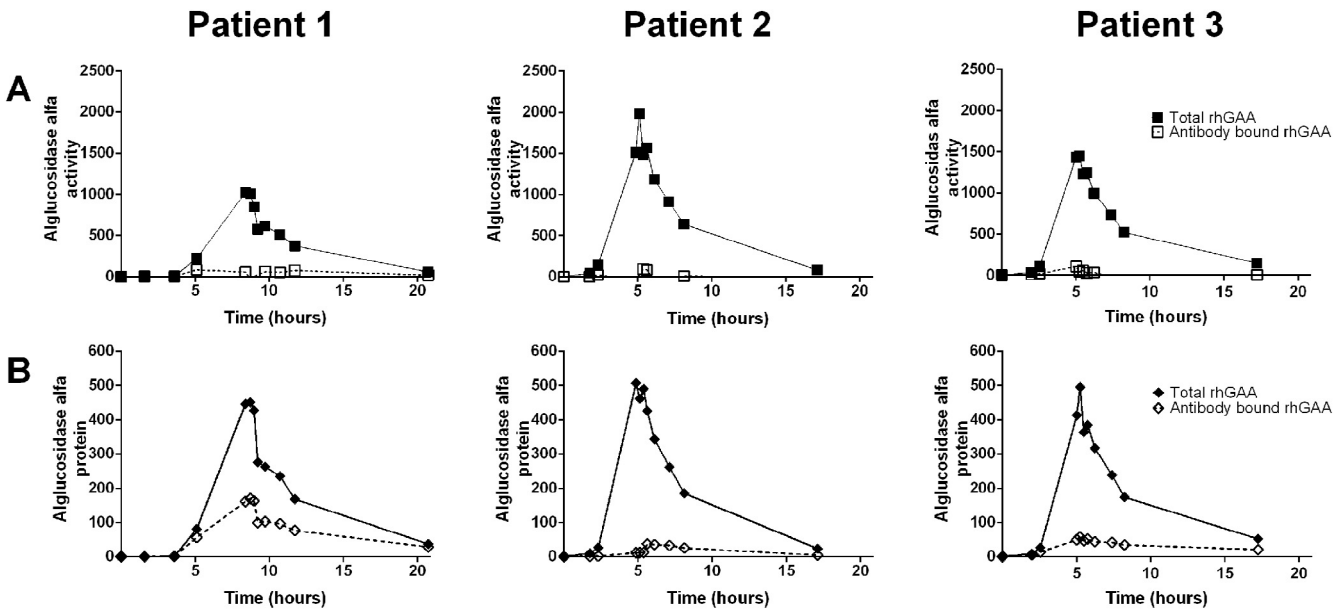

Figure 3. Pharmacokinetic profile of alglucosidase alfa activity and alglucosidase alfa protein during infusion with ERT. **A,** Alglucosidase alfa activity measured in serum before and during infusion with rhGAA. Closed symbols represent the total rhGAA enzyme activity. Open symbols represent serum that was first subjected to immunoprecipitation with protein A, which captures rhGAA bound to anti-rhGAA antibodies. The remaining rhGAA enzyme activity in the supernatant was measured and used to calculate the percentage of antibody-bound enzymatically active GAA. Results using protein G were similar. **B,** The experiment described in the upper row was now analyzed by mass spectrometry rather than enzyme activity and reports values for antibody-bound rhGAA protein irrespective of their enzymatic activity. Closed symbols represent the total rhGAA protein in sera; open symbols the antibody-bound rhGAA protein in sera.

To determine the effect of anti-rhGAA antibodies on rhGAA enzymatic activity during ERT, serum samples collected at several time points during infusion were used for immunoprecipitation with protein A-sepharose, followed by measurement of rhGAA activity in the supernatant (**Figure 3**, A). Sepharose only served as control. The analysis was performed at 20, 14, and 14 months of ERT for patients 1, 2, and 3, respectively. The amount of precipitated alglucosidase alfa activity in serum samples collected during infusion was close to zero in all 3 patients (**Figure 3**, A), suggesting that protein A-bound alglucosidase alfa was either enzymatically inactive or not present. To distinguish between these possibilities, we used mass spectrometry to detect alglucosidase alfa protein independent of enzyme activity, as reported by us recently.[25] **Figure 3**, B shows that the percentages of antibody-bound rhGAA protein were 37.7%, 2.7%, and 11.8% in patients 1, 2, and 3, respectively. We conclude that the anti-rhGAA antibodies in these patients preferentially bound to infused rhGAA protein that was enzymatically inactive.

To test the effect of anti-rhGAA antibodies on enzymatic activity in a cell-free assay, we incubated a fixed amount of rhGAA with increasing amounts of patient's serum, followed by immunoprecipitation with protein A and measurement of enzyme activity in the supernatant. Serum from the reference patient with known neutralizing effects of antibodies efficiently precipitated alglucosidase alfa activity (**Figure 5**; available at www.jpeds.com, gray line). In contrast, serum from patients 2 and 3 failed to precipitate alglucosidase alfa activity in vitro (**Figure 5**, squares and triangles). Patient 1 showed moderate capacity to precipitate alglucosidase alfa activity at 20 months of ERT (**Figure 5**, filled circles). However, when tested 7 months later (month 27), serum from patient 1 showed enhanced capacity to precipitate alglucosidase alfa activity (**Figure 5**, open circles), with an efficiency that was almost as strong as that of the reference patient. We conclude from these data that in patient 1, titers of antibodies with neutralizing effects increased from 20 to 27 months of ERT, and patients 2 and 3 did not show detectable neutralizing antibodies.

At study end, all 3 patients survived ventilator-free. LVMI z score decreased significantly in all 3 patients and normalized completely in patients 1 and 3. It was still slightly elevated in patient 2 at study end (LVMI 87 g/m^2, z score 3.5; SD 7.9, **Figure 4**, C),[21] and cardiac dilatation had disappeared and the shortening fraction normalized to 40% (data not shown). Motor function improved. All patients learned to walk independently at ages of 21, 18, and 17 months (patients 1, 2, and 3, respectively), and AIMS and Bayley Scales of Infant Development II scores increased accordingly (**Figure 4**, A and B). Nasogastric feeding tube could be withdrawn at ages of 15.8, 10, and 9 months. Oxygen supply and monitoring could be stopped after 3, 13.5, and 6 months of ERT. At the age of 29 months (24 months of ERT), patient 1 temporarily lost the ability to walk after a foot injury (**Figure 4**, B). At the age of

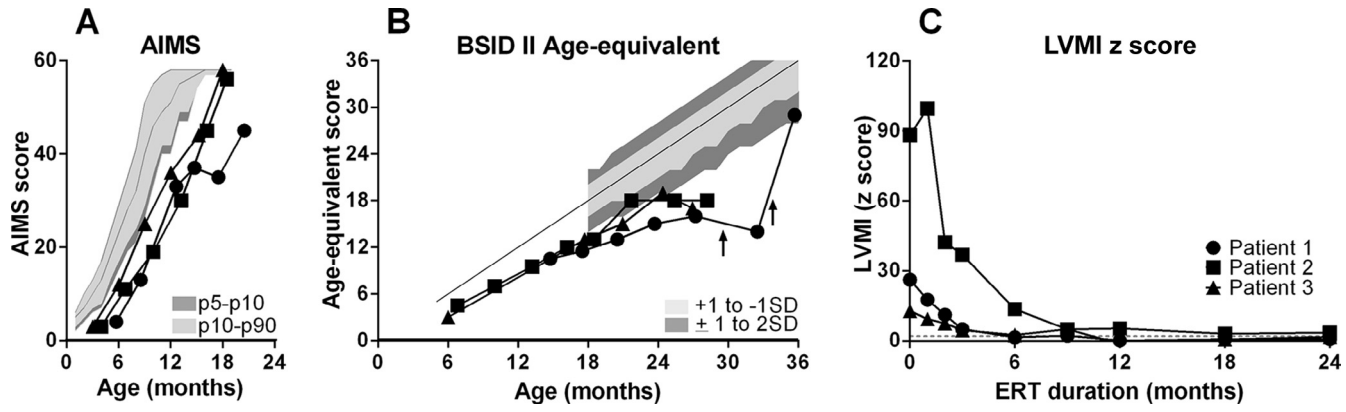

Figure 4. Effects of ERT and immunomodulation on clinical outcome. **A,** Motor function measured by AIMS; **B,** Motor function measured by Bayley Scales of Infant Development II. Raw scores were translated to age equivalent scores to compare patients. Patient 1 lost the ability to walk at the age of 29 months (*first black arrow*) and regained this ability at the age of 34 months (*second black arrow*). **C,** LVMI z score. Normal values depend on body surface area as described by Poutanen et al.[21] For a body surface area up to 0.75 normal values are 59.2 g/m^2 with an SD of 7.9 g/m^2.
BSID-II, Bayley Scales of Infant Development II.

34 months (28 months of ERT), he regained the ability to walk (albeit with lesser stability than before).

Discussion

The results of our study and other published studies on primary immunomodulation are summarized in **Table IV** (available at www.jpeds.com). Messinger et al described the effects of transient immunomodulation in 2 ERT naïve patients. One patient remained antibody-free for up to 2 years following cessation of transient RTX treatment, and the other patient developed a titer of 1:1500 at 7 months, which dropped to 1:400 after 18-24 months.[19] This group, using a slightly different transient immunomodulation protocol, also reported another patient who had low antibody titer of 1:3200 at 12 months.[31] On the other hand, a report by Elder et al found high antibody titers in the first patient 5 months after receiving transient immunomodulation.[29] This patient died 8 months later, and the authors decided to continue RTX treatment in the following 4 patients to suppress antibody formation. That same year, Banugaria et al published a decision flow chart in which patients with increasing antibody titers after transient immunomodulation received a second round of immunomodulation, which was applied to 2 out of 7 patients.[30] Broomfield et al reported on 9 patients receiving transient immunomodulation and only 1 developed an antibody titer.[15] In the present study, 2 out of 3 patients with our transient immunomodulation protocol developed very high antibody titers upon B cell recovery. Although other reports suggested that transient treatment with RTX plus MTX may induce long-term immune tolerance,[19,20,30,31] our results indicate that this protocol may induce a transient suppression of antibody formation rather than immune tolerance in certain patients. It should be noted that although all of these studies used RTX to eliminate B cells, variations existed with respect to additional treatments. For example, the duration and dosage of MTX treatment varied, and in some cases MTX was replaced by mycophenolate or sirolimus (**Table IV**). Also, the age at which ERT and immunomodulation was started varied between patients and ranged between 0.1 and 8 months of age (median 3.1 months). It is unknown to what extent these variations affect the immunomodulatory outcome. Clinical outcome of previously published patients that received immunomodulation was heterogeneous (**Table IV**). Of the 24 patients, 5 patients died (21%), and only 6 learned to walk (25%). In contrast, in the present study all 3 patients survived at study end and learned to walk. A likely important factor that contributed to the positive clinical outcome in the present study is the high dosage of rhGAA (40 mg/kg/week), which has previously been shown to have a beneficial effect compared with the standard dosage of 20 mg/kg every 2 weeks.[13] The patients in the previously published studies received either 20 mg/kg every 2 weeks or 20 mg/kg/week, Broomfield et al reported 2 patients in whom the dose was increased to 40 mg/kg/week.[15] Another factor to be considered is CRIM status. A negative CRIM status has been associated with a poor prognosis, reduced response to ERT, and tendency to form high antibody titers.[10-12] The majority of published patients in **Table IV** were CRIM negative, in line with the poor clinical outcome of these patients. However, patient 1 of the present study was also CRIM negative, yet survived and learned to walk, even though he started ERT the latest at the age of 5.8 months. Because antibodies in this patient only showed mild to moderate neutralizing effects (discussed below), we hypothesize that the high dosage of rhGAA may have been responsible for his relatively good clinical outcome at study end.

The very high antibody titers detected using ELISA showed mild to moderate (patient 1) to no (patients 2 and 3) neutralizing effects in vitro in cell-based assays (**Figure 2**), a cell-free assay (**Figure 5**), and on serum samples collected during

infusion with rhGAA (**Figure 3**, A). These assays have been shown to be capable of detecting neutralizing effects of antibodies, as highlighted previously.[17,18] Whether the immunomodulation protocol has influenced the nature or IgG subclasses of anti-GAA antibodies remains to be investigated in future analysis by comparing sera of patients who underwent immunomodulation or not. It is of concern that in patient 1, neutralizing effects increased from mild (~20%) in the first year to moderate (~40%) around study end (27 months of ERT). A limitation of the present study is the relatively short follow-up after cessation of RTX treatment and the low number of patients. Future work should be directed toward longer follow-up of formation of neutralizing antibodies and the efficacy of ERT in a larger cohort. To translate neutralizing effects measured in vitro to the situation in patients, one needs to take into account the dosage of ERT. At a higher dosage employed here, it is expected that neutralizing effects will be less severe compared with a regular dosage, as more antibody-free rhGAA will be available.

The inclusion of mass spectrometry as a read out for antibodies revealed a surprise: a considerable percentage of rhGAA protein was captured by antibodies during infusion in patient 1 (37%), but only a small fraction (2 %) of enzymatically active GAA was captured (comparing **Figure 3**, A and B). This suggests that infused rhGAA may undergo denaturation upon prolonged exposure to conditions present in the circulation. In agreement, we found that the half-life of rhGAA enzymatic activity in blood in vitro is only 100 minutes, while full length rhGAA protein remains detectable over prolonged periods.[32] In tissue culture medium and in the cellular fraction, we did not find evidence that antibodies from patients promoted degradation of rhGAA, although a larger study is required to confirm this. The ELISA assay cannot distinguish between antibodies directed toward native or denatured proteins, suggesting that ELISA titers per se are insufficient to predict potential deleterious effects of antibodies, and that additional analysis using neutralizing assays are required to more accurately link antibody formation to clinical outcome.

Although immunomodulation of ERT naïve patients with Pompe disease using a combination of RTX, MTX, and IVIG has been reported to be associated with prevention of antibody formation,[19,20] we showed that this treatment induces immune suppression during B cell depletion rather than tolerance in 2 out of 3 patients in our study. It will be important to optimize protocols that can provide long-term prevention of antibody formation without compromising general immunity in patients with Pompe disease treated with ERT. ■

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50 Years Ago in *The Journal of Pediatrics*

A Search for the Reservoir of Cytomegalovirus in Salivary Gland Tissue

ven Bensel RW, St Geme JW Jr. J Pediatr 1968;72:479-82

In this publication, ven Bensel and St Geme, Jr, described an unsolved mystery, 60 years after the description of cytomegalic inclusion disease and 10 years after the first isolation of cytomegalovirus (CMV): what was the manner of CMV acquisition beyond neonatal disease? They observed that the 80% seropositivity rate of persons older than 35 years of age described in the literature at the time was similar to that for herpes simplex. Recognizing that acquisition and probable latency occurred without symptoms, they postulated that the salivary glands could be a primary site of latency, with transmission and occasional disease the result of reactivation and salivary shedding.

Extracting salivary gland tissue immediately at necropsies, they sought microscopic evidence of cytomegalic inclusion disease and attempted isolation of CMV in tissue culture. A total of 100 unselected patients older than 10 years of age whose autopsies were performed at the University of Minnesota Medical Center over 1 year's time were subjects (31 acute accidental or surgical deaths, 47 deaths from cancer, and 22 from chronic systemic illnesses). Their results fit into a single sentence: They found no evidence of CMV cytopathic effect or positive culture in even 1 of 100 specimens.

Case closed? We know that CMV is shed in saliva, genital secretions, breast milk, and urine (the first 3 serving as major sources/vehicles of transmission) and is latent in mononuclear cells and several organs. Congenital, primary, and reactivation infection can lead to either, neither, or both silent infection or devastating disease, depending on the host. Saliva is a highly sensitive specimen source to screen newborns for congenital CMV infection via polymerase chain reaction testing. So why did the authors not find any reactivating salivary gland virus, with more than one half of subjects dying of cancer or another disease and thus presumably immunocompromised? This writer could speculate but really has no idea. Their premise was correct.

Reliving this time in clinical virology 50 years ago was special for this writer. Isolation of CMV in tissue culture, then, took a spectacular mean of about 30 days. The other more poignant memory was of Joseph W. St Geme, Jr, whom I had the privilege to know a bit, through Waldo E. Nelson and his team at St Christopher's Hospital for Children. Joe St Geme—a wonderful man and scientist and believer in young people and the future of pediatrics—lives on through his family, through the leadership award bearing his name that is given annually at the Pediatric Academic Societies Meeting, and in the stellar character of his son, Joseph W. St Geme, III, Chair of Pediatrics at the Children's Hospital of Philadelphia.

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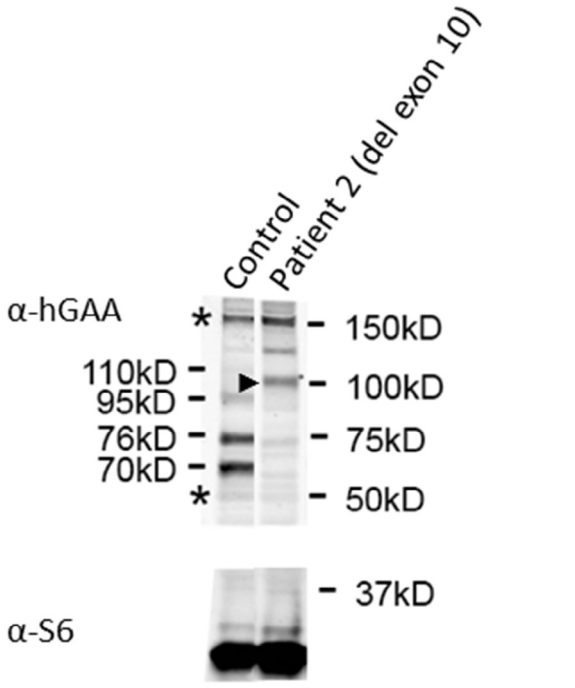

Figure 1. Immunoblot analysis of patient 2. Cell homogenates were prepared as described in Van Gelder et al 2015.[12] Equal amounts of protein were loaded per lane. The 2 lanes are control fibroblasts from a healthy individual and fibroblasts from patient 2. Patient 2 shows a specific band at ~105.8 kD, which is consistent with the size of the 110 kD precursor GAA protein minus 4.2 kD, caused by an in-frame skip of exon 10.[27]

Table II. Exome sequence analysis of 49 genes involved in hypertrophic cardiomyopathy applied in patient 2

Genes tested	
MYBPC3	GLA
MYH7	JPH2
TNNT2	LAMA4
TNNI3	LAMP2
MYL2	LDB3
TPM1	MIB1
TCAP	MYH6
CSRP3	MYOZ2
LMNA	MYPN
TNNC1	NEXN
ACTC1	PDLIM3
MYL3	PRDM16
PLN	PRKAG2
TAZ	RBM20
CALR3	TTR
TTN	VCL
ABCC9	CTNNA3
ACTN2	DSC2
ANKRD1	DSG2
BAG3	DSP
CAV3	JUP
CRYAB	PKP2
DES	SCN5A
EMD	TMEM43
FLH1	

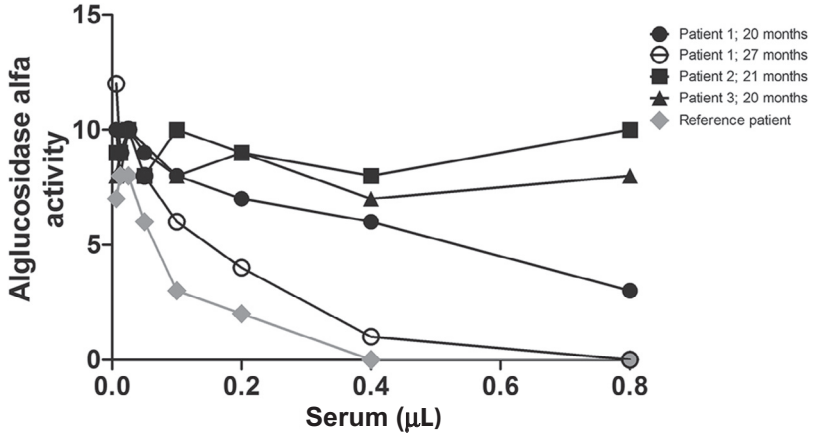

Figure 5. Antibody titers determined by immunoprecipitation under cell-free conditions. A fixed amount of rhGAA was incubated with various amounts of patient serum in vitro in the absence of cells, and anti-rhGAA antibodies were immunoprecipitated with protein A/G. The enzyme activity of rhGAA in the supernatant was used to determine the percentage of antibody-bound enzymatically active rhGAA (relative to control serum, using the reference patient with known neutralizing antibodies as described by de Vries et al in 2010).[18] Anti-rhGAA titers at time of analysis were 1:800 000 (patient 1, both time points); 1:200 000 (patient 2); and 1:6250 (patient 3).

Table III. IARs in patient 1

Type of IARs	Frequency
General discomfort	12
Fever	9
Shivering	7
Local erythema	8
Quivering lip	4
Hypotension	2
Palor	9
Tachycardia	9
Desaturation	1
Generalized erythema	2
Regurgitation	2
Swollen ear	1
Total infusions with IARs	15

Table IV. Overview of the literature of immunomodulation in patients with classic infantile Pompe naïve to ERT

Authors	Year	Pt	Allele 1	Allele 2	Age start ERT	Follow -up	Alive	Vent. free	Walks	IM treatment	IM duration	IM repeat	Time since last RTX	B cell recovery[§]	Peak titer (time after B cell recovery)[¶]	Neutralizing effects
Messinger et al[19]	2012	3	c.2560C>T	c.2560C>T	16 wk	24 mo	Yes	Yes	Yes	1A	5 wk	No	30 m	Yes	1:1600 (2 m)	ND
		4	c.1548G>A	c.525delT	15 wk	24 mo	Yes	Yes	Yes	1A	5 wk	No	17 m	Yes	0 (9 m)	ND
Elder et al[29]	2013	A	c.2560C>T	c.2560C>T	8 mo	11 mo	No	No	No	2A	5 wk	No	10 m	Yes	1:500 000 (5 m)	ND
		B	c.1396delG	c.1705dup	8 mo	36 mo	Yes	No[†]	No	2B	RTX ongoing	No	<12 wk	No	NA	ND
		C	c.925G>A	c.925G>A	2.75 mo	30 mo	Yes	Yes	No	2B	RTX ongoing	No	<12 w	No	NA	ND
		D	c.1548G>A	not found	6 mo	24 mo	Yes	Yes	No	2B	RTX ongoing	No	<12 wk	No	NA	ND
		E	c.1933G>A	c.2501_2502del	3 mo	22 mo	Yes	Yes	No	2C	RTX ongoing	No	<12 w	No	NA	ND
Banugaria et al[30]	2013	1	c.2608C>T	c.2608C>T	3.0 mo	101 wk	Yes	Yes	No	1E	5 wk	No	96 wk	Yes	0 (81 w)	ND
		2	c.546+2T>C	c.546+2T>C	4.1 mo	92 wk	Yes	NIV	No	1E	5 wk	No	87 w	Yes	0 (67 w)	ND
		3	c.236_246del	c.236_246del	2.4 mo	89 wk	Yes	Yes	Yes	1E	5 wk	No	84 w	Yes	0 (69 w)	ND
		4	c.525delT	c.2560C>T	0.1 mo	70 wk	Yes	No	No	1E	5 wk	No	65 w	ND	0 (NA)	ND
		5	c.2560C>T	c.2560C>T	0.5 mo	59 wk	Yes	NIV	No	1E	5 wk	Yes	24 w	No	1:6400 (before)	ND
		6	c.525_526delTG	c.525_526delTG	1 mo	51 wk	Yes	NIV	No	1E	5 wk	Yes	8 w	No	1:6400 (before)	ND
		7	c.2560C>T	c.2560C>T	1 mo	48 wk	No	No	No	1E	5 wk	No	43 w	Yes	0 (8 w)	ND
Stenger et al[31]	2015	1	c.2105G>T	c.2512C>T	1.2 mo	17 mo	Yes	Yes	Yes	1B	5 wk	No	39 w	Yes	1:3200 (39 w)	ND
Broomfield et al[15]	2016	6	c.525delT	c.2608C>T	4.7 mo	4.2 mo	No	No	No	1C	5 wk	No	~6 m	Yes	0 (>4-9 m)	ND
		7	c.2608C>T	c.2608C>T	6.6 mo	5.4 mo	Yes	Yes	No	1C	5 wk	No	~3 w	Yes	0 (0.6 y)	ND
		8	c.2237G>A	c.2237G>A	2.4 mo	9.6 mo	No	No	No	1C	5 wk	No	~9 m	Yes	0 (>4-9 m)	ND
		12	c.2078dup	c.2078dup	6.7 mo	8.9 mo	Yes	Yes	No	1C	5 wk	No	~16 m	Yes	0 (0.9 y)	ND
		14	c.2560C>T	c.2560C>T	18 d	17.5 mo	No	No	No	1C	5 wk	No	~17 m	Yes	0 (>4-9 m)	ND
		16	c.2237G>A	c.2237G>A	36 wk[*]	33 mo	Yes	Yes	Yes	1C	5 wk	No	~31 m	Yes	0 (>4-9 m)	ND
		17	c.2560C>T	c.2560C>T	5.2 mo	28.4 mo	Yes	Yes	Yes	1C	5 wk	No	~28 m	Yes	0 (1.8y)	ND
		19	c.525delT	c.2481+102_2646+31del	3.9 mo	32.9 mo	Yes	NIV	Yes[‡]	1C	5 wk	No	~32 m	Yes	1:12 800 (1.3 y)	ND
		22	c.877G>A	c.877G>A	2.2 mo	49.4 mo	Yes	NIV	No	1C	5 wk	No	~49 m	Yes	0 (2.5 y)	ND
This study	2017	1	c.525delT	c.525delT	5.8 mo	~24 mo	Yes	Yes	Yes	1D	MTX ongoing	No	23 m	Yes	1:800 000 (6.3 m)	mild/moderate
		2	c.1551+1G>A	c.1551+1G>A	4.2 mo	~24 mo	Yes	Yes	Yes	1D	MTX ongoing	No	23 m	Yes	1:6250 (7.3 m)	No
		3	c.525delT	c.2481+102_2646+31del	3.1 mo	~24 mo	Yes	Yes	Yes	1D	MTX ongoing	No	23 m	Yes	1:200 000 (6.3 m)	No

IM, immunomodulation; *NA*, not applicable; *ND*, not determined; *Neg*, negative; *NIV*, noninvasive ventilation; *Pos*, positive.

Immunomodulation (IM) treatment used per study: **1A** RTX 375 mg/m^2/dose for 4 doses; MTX 0.4 mg/kg/dose subcutaneous for 9-17 doses; IVIG 500 mg/kg administered once in patient 1. **1B** RTX 375 mg/m^2/dose for 4 doses; MTX 15 mg/m^2/dose oral for 9 doses; IVIG 400-500 mg/kg twice. **1C** RTX 375 mg/m^2/dose for 1-4 doses; MTX 0.4 mg/kg/dose subcutaneous for 10-18 doses. **1D** RTX 375 mg/m^2/dose for 4 doses; MTX 1 mg/kg/wk intravenous for the duration of follow up; IVIG 400 mg/kg monthly. **1E** RTX 375 mg/m^2/dose for 4 doses; MTX 0.4 mg/kg/dose subcutaneous for 9 doses; IVIG 400-500 mg/kg 5-6 times. **2A** RTX 750 mg/m^2/dose for 2 doses; Mycophenolate 300 mg/m^2/day oral; IVIG 500-1000 mg/kg monthly. **2B** RTX 375 mg/m^2/dose for 3 doses + repeat RTX every 12 weeks; Sirolimus 0.6-1 mg/m2/day oral; IVIG 500-1000 mg/kg monthly. **2C** RTX 750 mg/m^2/dose for 2 doses+ repeat RTX every 12 weeks; Sirolimus 0.6-1 mg/m2/day oral; IVIG 500-1000 mg/kg monthly.

*Patient 16 in Broomfield et al was born at 34 weeks of gestational age and ERT was initiated at 36 weeks of gestational age.

†Patient B became ventilator dependent after follow-up.

‡Patient 19 lost ability to walk 4 months after gaining it, within follow-up period.

§Ref 15: B cell recovery 4-9 months after last RTX; Personal communication, A. Broomfield.

¶Time after B cell recovery in ref 15 was 4-9 months after last RTX, time given here in age when tested.