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ORIGINAL RESEARCH ARTICLE

The Effect of Anti-drug Antibodies on the Bioavailability of Alglucosidase Alfa in Classic Infantile Pompe Disease

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Abstract

Background The impact of anti-drug antibodies (ADAs) on the bioavailability of recombinant human acid α-glucosidase (rhGAA) in classic infantile Pompe disease remains incompletely understood.

Objective The aim of this study was to investigate the effects of ADAs on the bioavailability of rhGAA in classic infantile Pompe disease.

Methods We analyzed 15 pharmacokinetic (PK) curves from 13 patients receiving rhGAA. High titers were defined as ≥ 1:31250. Alpha-glucosidase activity in plasma was measured using protein A beads to precipitate ADA-bound rhGAA, and sepharose as control. Neutralizing effects were assessed in fibroblasts and culture medium after incubation with patient sera and a fixed amount of rhGAA. Peak activity (C_{max}) and area under the curve (AUC) were calculated by non-compartmental analysis.

Results rhGAA bioavailability was affected in six of nine high-titer patients ($n = 1$, 1:31,250; $n = 5$, ≥ 1:156,250). AUC was reduced by 11–52% in the protein A assay compared with sepharose ($n = 5$). Fibroblast uptake studies demonstrated neutralizing effects with intracellular enzyme activity reduced to 47–71% ($n = 5$). No ADA effect was detectable in three patients. At the group level, high-ADA patients showed significantly lower C_{max} in both assays and a lower AUC in the protein A assay compared with non-high-ADA patients. Slower infusion schemes, as management for infusion-associated reactions, correlated inversely with C_{max} ($R = -0.64$), but not AUC. Immunomodulation eliminated the effects of ADAs, as shown in two patients.

Conclusions High ADAs have heterogenous effects on rhGAA bioavailability. Titers ≥1:156,250, mostly, had a measurable effect, capturing up to > 50% of infused rhGAA. A high titer, per se, did not imply neutralizing effects. Where available, functional assays, including PK curves and uptake studies, may contribute to characterize ADA effects.

1 Introduction

Pompe disease (glycogen storage disease type II, OMIM: #232300) is caused by the deficiency of the lysosomal enzyme acid-alpha-glucosidase (GAA). The phenotype is broad, ranging from the most severe spectrum of the disease, classic infantile Pompe disease, to the less severe form, late-onset Pompe disease, which may present in children as well as in adults [1]. Since 2006, enzyme replacement therapy (ERT) with recombinant human acid α-glucosidase (rhGAA, alglucosidase alfa) has been available and has significantly improved patient outcomes. The response to treatment varies among individuals, and the main factors in patients with the classic infantile form are dose of ERT [2–6], age at start of treatment [7] and immune response to ERT. Most ERT-treated classic infantile Pompe patients develop anti-drug antibodies (ADAs), which can antagonize the effect of therapy [8–10]. Historically, high antibody titers (≥ 1:31,250/≥ 1:12,800, depending on the type of assay [11]) have been attributed to the complete absence of residual GAA protein, known as 'cross-reactive immunological material (CRIM) negative'; to date, it is known that 30–40% of CRIM positive classic infantile patients, who produce (some) inactive GAA protein, also develop high titers [8, 10, 12–15]. Among these patients, it remains difficult to predict who will develop high ADAs. Potential risk factors

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Key Points

Anti-drug antibodies may have an effect on the available amount of enzyme replacement therapy reaching target tissues in patients with classic infantile Pompe disease, but their effects vary widely.

Using complementary assays, this study shows that antibodies can interfere with treatment in multiple ways, including binding to the infused enzyme, reducing its uptake by cells, lowering total enzyme activity, and contributing to infusion-associated reactions, ultimately decreasing the amount of therapy available for tissue uptake.

These mechanisms may also be relevant to other lysosomal storage diseases treated with enzyme replacement therapy. In addition, the study shows that, when applied correctly, immunomodulation can eliminate antibody-related effects and restore treatment effectiveness.

described include older age at start of therapy, higher doses of ERT at start [8, 15–18], and specific human leukocyte antigens (HLAs) [19]. Immunomodulation protocols have been adopted to either prevent the formation or to eliminate ADAs, using drugs such as rituximab (RTX), methotrexate (MTX), immune globulins (Ig) and bortezomib (BTZ) [2, 8–10, 14, 16, 18, 20].

The clinical course of patients who develop high ADAs is currently unpredictable; while some present a rapid clinical decline, in other cases the clinical course seems to be less affected by the high titers. Earlier we concluded that the antibody titer alone was not a sufficient indicator of the deleterious effect of antibodies, as ADAs can bind to the active site, but also other parts of the GAA molecule, and also that their effect relates to the dose of ERT applied [17]. High ADAs can reduce the availability of ERT to the target tissues and/or provoke infusion-associated reactions (IARs), which are managed through pre-medication with corticosteroids and antihistamines and modified infusion schemes, with slower infusion rates and an overall longer duration. In light of this complexity, we evaluated the effects of antibodies on the bioavailability of rhGAA by performing pharmacokinetic (PK) curves and studying rhGAA uptake in cells in a large group of classic infantile patients.

2 Methods

2.1 Study Design and Participants

Patients with classic infantile Pompe disease were eligible for the study, defined by the onset of symptoms of muscle weakness within 6 months after birth, the presence of hypertrophic cardiomyopathy, confirmed deficiency of acid alpha-glucosidase in leukocytes and/or fibroblasts, and two very severe variants in the GAA gene according to the Pompe Variant Database (http://www.pompevariantdatabase.nl) [21, 22], if a pharmacokinetic curve had been obtained.

All participants were enrolled in the prospective protocol MEC-2007-103, approved by the Medical Ethical Committee of the Erasmus MC University Medical Center.

2.2 Clinical Data

The following clinical data were collected: sex, GAA variants, CRIM status, age at start of treatment, age at PK assessment, infusion scheme, occurrence of infusion-associated reactions (IARs), antibody titer at the time of PK analysis and neutralizing assays. In two patients (patients 3 and 4), PK curves were obtained before immune tolerance induction (ITI) (A) in the context of high sustained antibody titers (secondary ITI) and during maintenance with long-term RTX (B), at intervals of 21 and 24 months, respectively. Secondary immunomodulation, consisting of RTX, MTX, BTZ, and intravenous immunoglobulin (IVIG), followed by long-term RTX maintenance, was administered according to the protocol shown in Fig. 1.

2.3 Pharmacokinetic Curves

Fifteen PK curves in 13 patients were performed between 2009 and 2024. Reasons to perform a PK curve were: (1) high ADAs; (2) change of ERT dose and/or frequency; (3) evaluation of the effects of ITI. The results of four PK curves have been published [3, 17]. Blood samples were taken at fixed timepoints before, during and after the infusion of 40 mg/kg rhGAA. The timepoints varied slightly over the study period. The start and end times of infusion as well as the exact times of blood collection were recorded:

- Patient 1, 2, 3B, 4B, 5: before start of ERT, at 30 mg/kg dose infused, end of infusion, at +15, +30, +60, +120 min, +4, +8, +16, +24 h.
- Patient 3A, 4A, 6: before ERT, 10 mg/kg dose, 20 mg/kg dose, 30 mg/kg dose, end of infusion, +15, +30 min, +1, +2, +3, +6, +8, +18 h.
- Patient 7, 8, 9: before ERT, 15 min before first step, 15 min before third step, 15 min before fourth step, 15 min before end, end of infusion, +15, +30, +60 min, +2, +3, +12 h.
- Patient 11, 12, 13: before ERT, end of infusion, +15 min.
- Patient 10: before ERT, +2, +3 h from start of ERT, 15 min before end of ERT, end of ERT, +15, +30, +60, +120 min.

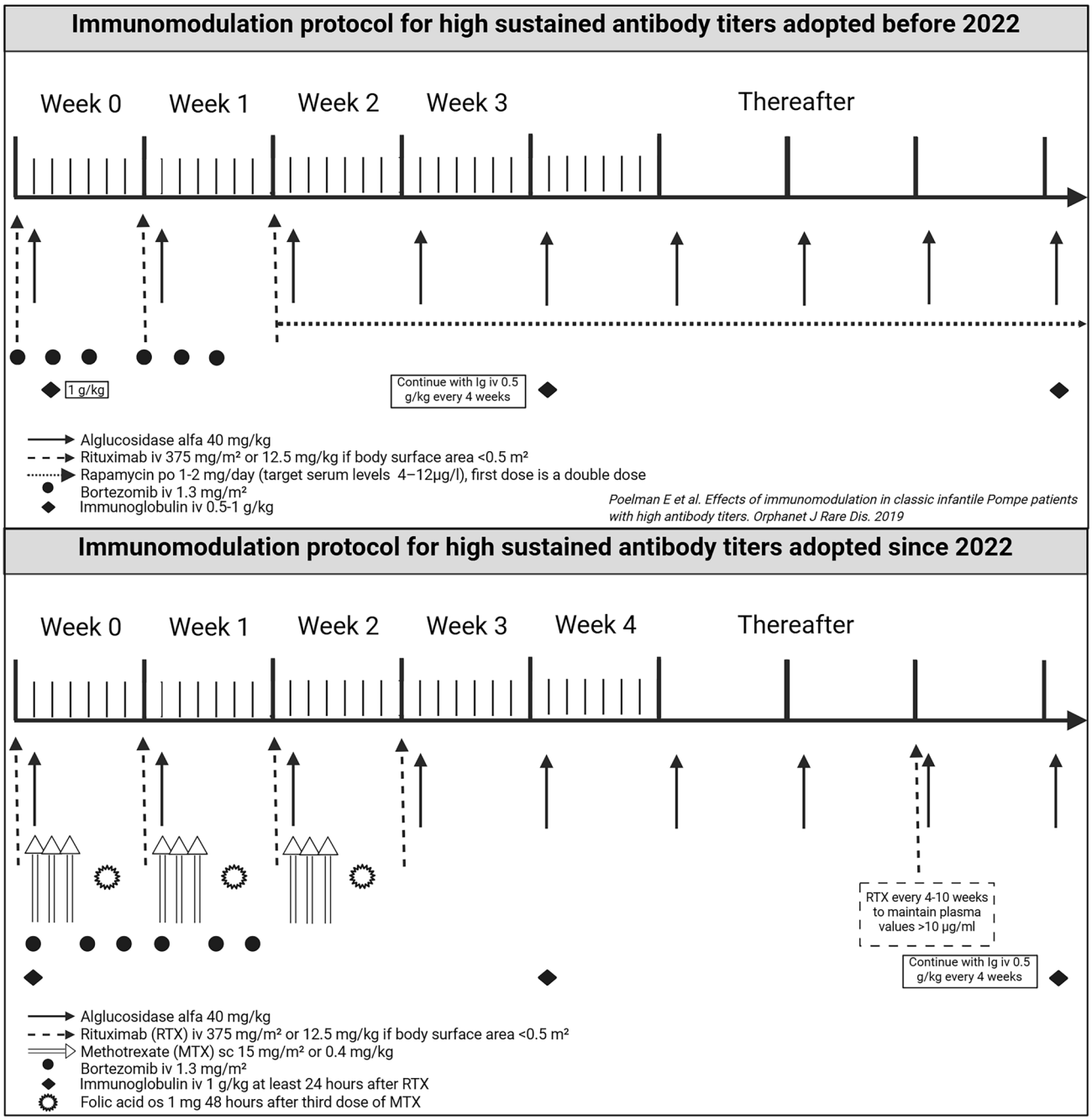


Fig. 1 Immunomodulation protocol adopted in our Center before [18] and after 2022. Such protocol applies to enzyme replacement therapy–experienced patients in the context of high sustained antibody titers (secondary immunomodulation). Since 2020, we have administered immunoglobulins at least 24 h after rituximab infusion [23]. *BTZ* bortezomib, *Ig* immune globulins, *iv* intravenous, *MTX* methotrexate, *RTX* rituximab, *sc* subcutaneous, *po* oral administration. Figure created in BioRender.com

During the PK study, blood samples were collected from the central catheter, if present (with proper flushing of the line), or from a peripheral vein. The samples were collected and stored in the fridge at + 4 °C prior to centrifugation at 2000 rpm at room temperature to isolate the supernatant, which was then frozen at − 80 °C until analysis.

2.4 Alglucosidase Alfa Infusion

The standard infusion scheme applied at our Center for classic infantile Pompe disease consists of four steps: 0.28 mg/kg/h for 60 min, 0.82 mg/kg/h for 60 min, 3.80 mg/kg/h for 30 min, 15.00 mg/kg/h for the remainder of the infusion, for

a total of approximately 5 h. If patients experience IARs, we administer premedication and personalize the infusion scheme [24], by reducing the initial rates and, in some patients, diminishing the final rate below 15 mg/kg/h, both leading to a prolongation of infusion duration.

2.5 Alpha-Glucosidase Activity Measurement

To determine the percentage of antibody-bound enzyme in the blood, plasma samples were incubated in the presence of protein A beads, which bind antibody-bound alglucosidase alfa, and, in parallel, in the presence of sepharose beads only, as a control. Two-fold serial dilutions were made starting from 50 to 3200-fold in sodium-phosphate buffered saline (10 mM PBS, pH 7.0) containing bovine serum albumin (BSA, Sigma) in a concentration of 1 mg/mL (PBS/BSA). A 50-µL solution of alglucosidase alfa (total activity approximately 10 nmol 4 MU/h) in PBS/BSA was mixed with 10 µL of diluted serum and 20 µL of a 1:1 suspension of Protein A sepharose CL-4B beads in PBS and incubated under continuous agitation for 1 h at room temperature. The beads were then removed by centrifugation (14,000*g*) and the amount of antibody-bound alglucosidase alfa was calculated by measuring the enzyme activity in 10 µL of the supernatant using 20 µL of 4-methylumbelliferyl-α-D-glucopyranoside (MUGlc, Sigma) substrate and expressed as nmol/10 µL/h, as described before [3, 15, 17].

2.6 Antibody Titers

Blood samples for ADA determination were collected before the infusion on the day of the PK assessment. Standardized antibody analysis was performed in duplicates by an enzyme linked immunosorbent assay (ELISA) [17]. A high antibody titer was defined as ≥ 1:31,250.

Table 1 Characteristics of the patients included in the study

Patient	Sex	GAA Variants (NM_001079803.3)	CRIM	Age at start of ERT (months)	ITI history	Year of PK curve	Age at PK curve (years)
1	F	c.2104C>T/c.2104C>T	+	4.6		2024	8.4
2	M	c.2481+102_2646+31del/c.1597T>C	+	7.5	Primary [17]	2024	6.6
3	F	c.525del/c.525del	−	2.0	Secondary [18] (2.3 years) Secondary with long-term RTX (8.5 years)	2022; 2024	8.4 (A); 10.4 (B)
4	F	c.634G>T/c.1051del	−	11.4	Primary (MTX only) Secondary with long-term RTX (19 months)	2022; 2024	1.5 (A); 3.3 (B)
5	F	c.655G>A/c.655G>A	+	3.0		2024	2.5
6	F	c.525del/c.525del	−	4.6	Primary [17]	2022	8.3
7	F	c.1551+1G>A/c.1551+1G>A	+	4.3	Primary [17]	2014	1.5
8	M	c.525del/c.525del	−	5.9	Primary [17]	2014	2.1
9	M	c.525del/c.2481+102_2646+31del	+	3.2	Primary [17]	2014	1.4
10	M	c.525del/c.1933G>T	+	1.2		2009	5.5
11	M	c.525del/c.2481+102_2646+31del	+	3.8		2012	1.9
12	F	c.2104C>T/c.379_380del	+	5.0		2012	2.1
13	F	c.2481+102_2646+31del/c.2481+102_2646+31del	+	2.4		2012	4.2

The primary ITI regimen, administered to enzyme replacement therapy–naïve patients, has been described by Poelman et al. [17], as has the secondary regimen used in ERT-experienced patients with high sustained antibody titers [18]. An optimized secondary scheme, including maintenance with long-term RTX, has been implemented in our Center since 2022 and is depicted in Fig. 1. In two patients (patients 3 and 4), pharmacokinetic curves were obtained before secondary ITI (A) and during maintenance with long-term RTX (B), at intervals of 21 and 24 months, respectively

CRIM cross-reactive immune material, *ERT* enzyme replacement therapy, *GAA* acid-alpha-glucosidase, *ITI* immune tolerance induction, *MTX* methotrexate, *PK* pharmacokinetic, *RTX* rituximab

2.7 Neutralizing Activity

Neutralizing effects of antibodies were measured in duplicates, as described previously, in patients with a high antibody titer and a full PK curve [8, 17, 25]. Fibroblasts from a patient homozygous for the c.525del variant fully deficient in acid alpha-glucosidase production were seeded in 24-well tissue-culture plates and maintained at 37 °C in Ham's F10 medium supplemented with 10% FCS (fetal calf serum) and antibiotics. To measure the uptake of alglucosidase alfa, we added piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) to the medium in a final concentration of 3 mM to make the medium slightly acidic (pH 6.8). The enzyme was added in an amount equivalent to 200 nmol MUGlc/h per 200-µL medium. Finally, 20 µL of the patients' serum were added. Uptake of alglucosidase alfa was measured after 24 h in the medium and in cell homogenates. As control, the same experiment was performed without the addition of 20 µL of the patients' sera. GAA activity was expressed as percentage of control [18]. ADAs were defined as neutralizing if the enzyme activity was ≤ 75% [26].

2.8 Statistics

To determine the individual PK parameters, non-compartmental analysis (NCA) was performed to calculate the peak activity (C_{max}) and area under the curve extrapolated to infinity (AUC∞) with the R package 'PKNCA version 0.11.0' [27]. Pearson/Spearman correlations were studied for numerical data (C_{max}, AUC, duration of infusion, blood volume), while independent sample T-test/Mann Whitney *U* Test for categorical data: infusion scheme (standard/personal), antibody titer (high ≥ 1:31,250, non-high < 1:31,250), IARs (present/not present).

Table 2 Effects of anti-drug antibodies on the bioavailability of recombinant human acid α-glucosidase-(rhGAA) in 13 patients with classic infantile Pompe disease

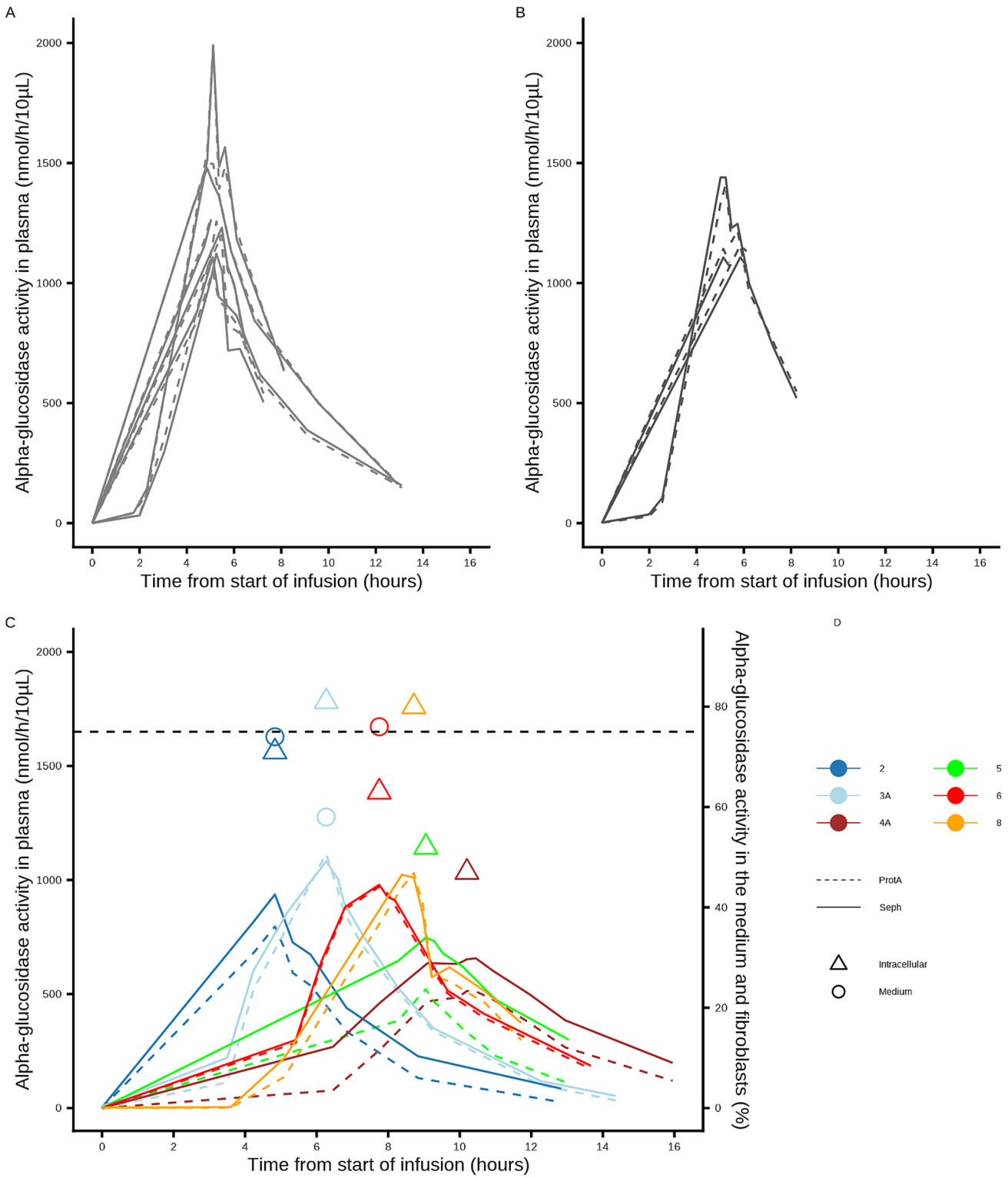
Patient	CRIM	IAR before PK curve	Duration infusion (minutes)	ADA at PK curve	C_{max} difference sepharose/protein A	AUC difference sepharose/protein A	rhGAA activity in the medium (% of control)	rhGAA activity in cell lysate (% of control)	Hypothesized effect of ADAs*
1	+	No	306	1:6250	5%	5%			
2	+	No	290	1:156,250	**15%**	**30%**	**74%**	**71%**	1 + 2 + 3
3A	−	No	376	1:156,250	− 3%	**11%**	**58%**	81%	1 + 3
3B		No	292	1:1250	− 1%	− 1%	90%	100%	
4A	−	Yes	597*	1:156,250	**22%**	**40%**	92%	**47%**	1 + 3 + 4
4B		No	329	1:1250	2%	3%	92%	95%	
5	+	Yes	543*	1:156,250	**30%**	**52%**	92%	**52%**	1 + 3 + 4
6	−	Yes	465*	1:31,250	1%	4%	**76%**	**63%**	3 + 4
7	+	No	307	1:6250	0%	0%	> 92%	105%	
8	−	Yes	523*	1:781,250	− 1%	**15%**	> 92%	**80%**	1 + 2 + 4
9	+	No	314	1:156,250	2%	2%	> 92%	95%	No effect
10	+	No	315	1:1250	− 12%	− 16%			
11	+	No	308	1:31,250	− 3%	NA			No effect
12	+	No	285	1:6250	− 1%	NA			
13	+	Yes	351*	1:31,250	− 5%	NA			4

In two patients (patients 3 and 4), pharmacokinetic curves were obtained before secondary immune tolerance induction (ITI) (A) and during maintenance with long-term rituximab (B), according to the protocol shown in Fig. 1. The difference in C_{max}/AUC between the sepharose and protein A assay is expressed as: [100 − (protein A value/sepharose value)] * 100. Neutralizing activity of antibodies was measured in patients with high antibody titers; plasma samples were incubated with fibroblasts derived from a classic infantile patient and a fixed amount of rhGAA. The effect of antibodies on enzyme activity was studied in the medium and in cell lysate. For the six patients with an effect of antibodies on the bioavailability of rhGAA, we estimated the effect of antibodies by combining the results obtained from the PK curves, uptake studies in fibroblasts and the presence of IARs

ADA anti-drug antibody, *AUC* area under the curve, C_{max} peak activity, *CRIM* cross-reactive immune material, *IAR* infusion associated reaction, C_{max} maximum concentration, *AUC* area under the curve, *NA* not available, *PK* pharmacokinetic, *rhGAA* recombinant human acid α-glucosidase

Bold formatting denotes assays in which a measurable effect of antibodies was detected

*Legend: 1 = precipitation of rhGAA (PK curve); (2) reduced uptake (cell lysates); (3) reduced total activity (low PK curve with a standard scheme and/or reduced activity in the medium); (4) infusion-associated reactions. * indicates personalized infusion schemes


A
B
C
D
Alpha-glucosidase activity in plasma (nmol/h/10µL)
Time from start of infusion (hours)
Alpha-glucosidase activity in the medium and fibroblasts (%)
2
3A
4A
5
6
8
ProtA
Seph
Intracellular
Medium

◂**Fig. 2** Alpha-glucosidase activity measured in plasma with the addition of protein A (*dashed line*) and sepharose (*continuous line*) beads after the infusion of 40 mg/kg of recombinant human acid α-glucosidase (rhGAA) in classic infantile Pompe disease. **A** Pharmacokinetic (PK) curves of six patients with a low/intermediate anti-drug antibody (ADA) titer (Table 2). **B** PK curves of three patients with a high ADA titer but no measurable effect of ADAs on the bioavailability of rhGAA (Table 2). **C** PK curves and fibroblast uptake studies in six patients with an effect of ADAs on the bioavailability of rhGAA. *Right y-axis:* effect of patient sera on α-glucosidase activity in medium (*circles*) and fibroblast lysates (*triangles*). Control enzyme activity was set at 100%; a neutralizing effect was defined as ≤ 75% residual activity (*dashed horizontal line*). A 'dissociation' (i.e. a separation between the protein A and sepharose curve) was found in patients 2, 3A, 4A, 5 and 8. A reduced enzyme activity in the medium was observed in patients 2 and 3A and was borderline in patient 6, whereas intracellular activity was reduced in patients 2, 4A, 5, and 6 among the patients tested (see Table 1). In patients 4, 5, 6, and 8, the peak (C_{max}) was delayed due to the personalized slower infusion scheme

3 Results

3.1 Patient Characteristics

Since 2009, 15 pharmacokinetic (PK) curves have been performed in 13 patients. Patient characteristics (i.e. their GAA variants, CRIM status, sex, age at start of treatment and age at PK curves) are provided in Table 1. A summary of the clinical course of the patients is provided in the Electronic Supplementary Material (ESM). All patients were clinically diagnosed as no newborn screening program is yet available in the Netherlands. The median age at diagnosis was 3.3 months (range 0.7–9.5). Five of the 13 patients were treated with an immunomodulation scheme in the ERT-naïve setting (primary immunomodulation), consisting of a 4-week scheme including RTX, MTX and IVIG, as described by Poelman et al. [17]. Patient 4 had received multiple doses of MTX at the time of start of treatment (see ESM). Patient 3 was treated at the age of 2.3 years with a secondary immunomodulation protocol in the context of high sustained antibody titers in use at that time (ESM and Fig. 1, before 2022). Subsequently, patients 3 and 4 were treated with an optimized protocol of secondary ITI including long-term RTX maintenance (Fig. 1 after 2022) and the PK curve was performed before ITI (A) and (B) during long-term RTX maintenance.

The PK curves were performed at ages ranging from 1.4 to 10.4 years. Nine of the 13 patients had a high titer at the time of the PK curve, of whom four were CRIM negative and five were CRIM positive. Three had a titer of 1:31,250, 5 of 1:156,250 and one of 1:781,250 (Table 2). Alpha-glucosidase activity was measured both in the presence of protein A beads, which precipitate immunoglobulin G (IgG) immune complexes (ADAs + rhGAA), and with sepharose beads as a control.

The median peak enzyme activity (C_{max}) was 1142 nmol/10 µL/h (range 513.4–1994.3) in the protein A assay and 1107 nmol/10 µL/h (range 656.6–1987.4) in the sepharose assay. The AUC could be estimated in 12 patients. The median AUC was 5540 nmol/10 µL (range 3352–9500) in the protein A assay and 6072 nmol/10 µL (range 4627–9407) in the sepharose assay.

3.2 Pharmacokinetic Curve Findings

Nine of the 13 patients, in whom a PK assessment was obtained, had a high anti-rhGAA titer. We found that high ADAs had an effect on the bioavailability of rhGAA in five of nine patients, as reported in Table 2 and shown in Fig. 2, Panel C, as a 'dissociation', that is, a difference between enzyme activity measured with sepharose (continuous line) and after washing away antibody-bound enzyme via protein A beads (dashed line). In three patients (2, 4A, 5), all of whom had a titer of 1:156,250, AUC was reduced in the protein A assay by 30%, 40% and 52%, respectively, in comparison with the sepharose assay. In the same patients, the protein A C_{max} was also lower than the sepharose by 15, 22, and 30%, respectively. Patient 3A (titer 1:156,250) and patient 8 (titer 1:781,250) had an 11% and 15% lower AUC in the protein A assay in comparison with the sepharose one, while C_{max} was similar. The remaining four patients with a high titer, of whom one had a titer of 1:156,250 (patient 9) and three had a titer of 1:31,250, did not show differences between alpha-glucosidase activity measured with the addition of the two types of beads (Fig. 2, Panel B). The six additional PK curves were performed in patients who did not have a high titer at that time; no effect of ADAs was found (Fig. 2 Panel A).

3.3 rhGAA Uptake Findings

Ten alpha-glucosidase uptake studies in fibroblasts to assess neutralizing activity were performed in eight patients, of whom seven had a high titer (Table 2). An inhibitory effect of antibodies was observed on the enzyme activity in the medium of patients 2, 3A and was borderline in patient 6; a neutralizing effect on uptake was evident in cells of patients 2, 4A, 5, and 6 (Table 2 and Fig. 2, Panel C). Four of the five patients (2 CRIM negative and 2 CRIM positive) had an antibody titer of 1:156,250 and one (CRIM negative) of 1:31,250. Patient 9, with a titer of 1:156,250, did not present a neutralizing effect and in patient 8, with the highest titer of 1:781,250, only the enzyme activity within the cell lysates was reduced to 80%. In the remaining three studies,

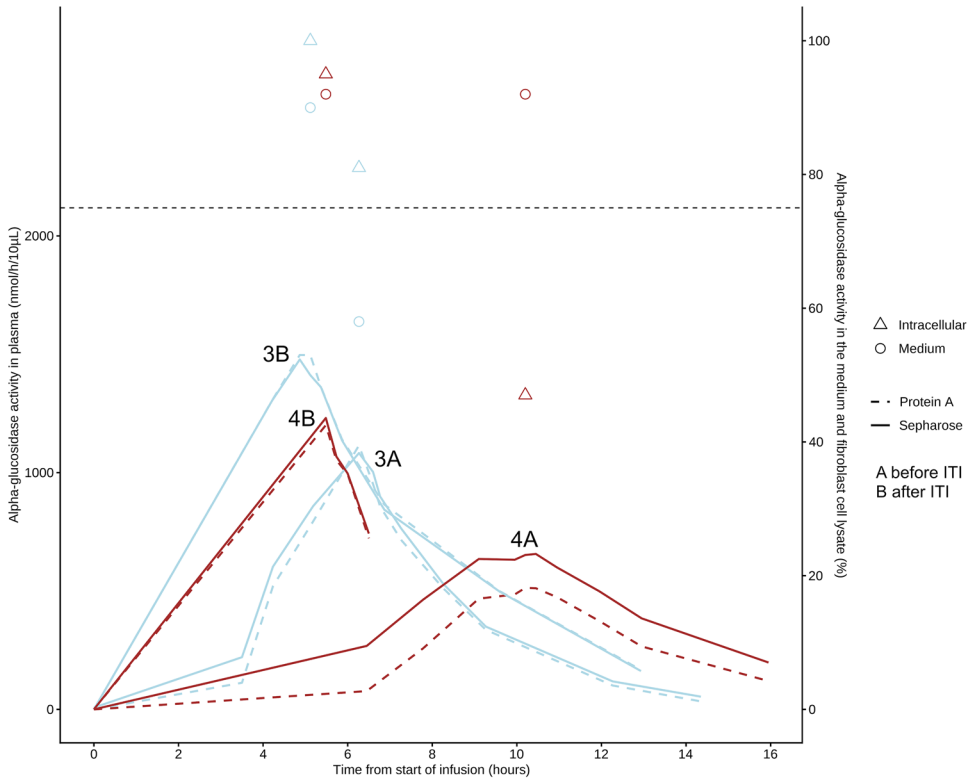


Fig. 3 Immunomodulation reverses the effect of high antibody titers. Alpha-glucosidase activity measured with the addition of protein A (*dashed lines*) and sepharose (*continuous lines*) (left y axis). In patients 3 and 4, PK curves were obtained before immune tolerance induction (A) and during long-term rituximab (RTX) (B), at intervals of 21 and 24 months, respectively. Immunomodulation, consisting of RTX, methotrexate (MTX), bortezomib (BTZ), and intravenous immunoglobulin (IVIG), was administered according to the protocol shown in Fig. 1. On the right y axis, alpha-glucosidase activity is studied with the addition of patients sera in the medium (*circle*) and cell lysate (*triangle*). Control is set at 100%. Neutralizing activity of antibodies is defined as alpha-glucosidase activity ≤ 75% in cell lysates or medium (*dashed horizontal line*)

performed in patients who did not have a high ADA titer, no effect on uptake was found.

3.4 Immunomodulation Reversed the Effects of High ADAs

In two patients (patients 3 and 4), PK curves were performed before secondary ITI and during maintenance with long-term RTX, 21 and 24 months apart, respectively (Fig. 3). Secondary immunomodulation, consisting of RTX, MTX, BTZ, and intravenous Ig (Fig. 1), followed by long-term RTX, reduced the antibody titer from 1:156,250 to 1:1250 and eliminated the effect of high antibodies on the bioavailability of rhGAA; after ITI, the dissociation between alpha-glucosidase activity in the protein A and sepharose assay disappeared in both patients (Table 2). The in-vitro neutralizing effects of high ADAs were also eliminated (Table 2 and Fig. 3). Notably, after ITI, IARs disappeared in both patients, allowing shorter infusion durations and higher infusion rates.

3.5 Analysis of PK Parameters at a Group Level

We analyzed C_{max} and AUC at a group level. The high ADA patients ($n = 9$) had a significantly lower C_{max} in both assays (protein A $p = 0.018$, sepharose $p = 0.007$) in comparison with patients with an intermediate or low titer ($n = 6$), and a significantly lower AUC in the protein A assay ($p = 0.048$), but not in the sepharose assay ($p = 0.52$), compared with those without a high titer (Fig. 4, Panel A and C). The significant C_{max} difference in the protein A and sepharose assay was mostly influenced by the infusion scheme. Indeed, high ADAs may lead to infusion-associated reactions, which are managed by administering premedication drugs and/or adjusting the infusion schedule.

Five of nine patients (patients 4A, 5, 6, 8, 13), all with a high titer, had a previous history of IARs; to manage these, the infusion scheme had been modified with slower infusion rates and a longer infusion duration (Table 2). All other patients, including four with a high titer, had a standard infusion scheme. We found an inverse correlation ($R - 0.64$ sepharose / $- 0.57$ protein A) between the duration of the infusion and C_{max}; in addition, although not significant due to the small numbers of patients in the subgroups, the C_{max} of patients with high ADAs and a personal infusion scheme was generally lower in comparison with those with high ADAs and a standard scheme in the protein A assay (Fig. 4, Panel B). In contrast, AUC was lower in patients with high ADAs in comparison with low/intermediate ADAs, as previously mentioned, but no difference was found among patients with high ADAs and standard or personal infusion scheme in either the protein A or sepharose assay (Fig. 4, Panel D).

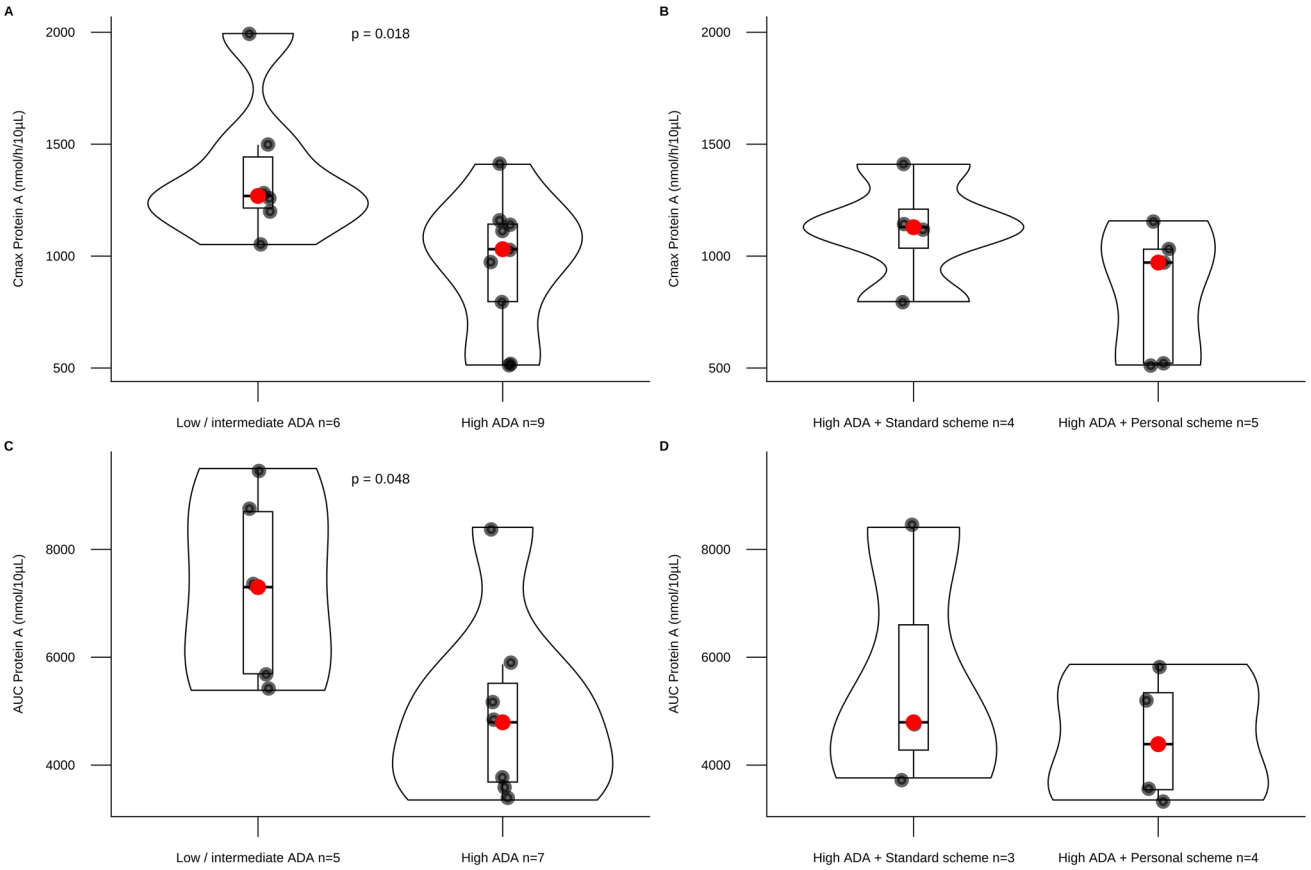


Fig. 4 Group-level comparison of peak enzyme activity (C_{max}) and area under the curve (AUC) between patients with low/intermediate and high anti-drug antibody (ADA) titers (Panels A and C), and between patients with high ADA titers receiving a standard versus individualized slower infusion regimen (Panels B and D). In the protein A assay, patients with high ADA titers showed lower C_{max} and AUC compared with patients with low/intermediate titers ($p = 0.018$ and $p = 0.048$, respectively). Among patients with high ADA titers, C_{max} was generally lower in those receiving an individualized slower infusion regimen compared with a standard infusion regimen, whereas AUC was comparable between groups. The *red dot* represents the group median

4 Discussion

In this study, we evaluated the effects of antibodies on the bioavailability of rhGAA in classic infantile Pompe disease by combining pharmacokinetic curves and fibroblast studies. This combined approach allowed us to identify several key effects of ADAs, including (1) binding of ERT immediately after infusion; (2) inhibition of uptake by cells; (3) interference with total measurable ERT activity; and (4) the occurrence of infusion-associated reactions.

The different assays we used were each informative for different types of effects. For example, the PK curves, performed in the presence of protein A and sepharose beads, inform us about the amount of active enzyme that is actually bound by antibodies immediately after infusion (effect 1). The assays on fibroblasts, instead, provide different information. By adding a fixed amount of rhGAA to the medium of cells, with and without the addition of patient sera, we can determine whether ADAs interfere with enzyme uptake by cells (effect 2) and/or overall enzyme activity (effect 3). This latter effect may be also detected by the PK curves as an overall lower enzyme activity, both in the protein A and sepharose assays, but it may be masked by a prolonged infusion duration, which is also associated with a lower C_{max}.

The present data provide direct evidence that anti-drug antibodies can have an impact on the bioavailability of rhGAA in patients with classic infantile Pompe disease. Such impact was observed exclusively in patients with a high titer. In contrast, no impact of ADAs on either pharmacokinetics or fibroblast-based assays was detected in patients with titers up to 1:6250. Notably, we also identified three cases with high titers in whom no measurable effects of ADAs on rhGAA were observed at the time of measurement (although fibroblast studies were not available for two of these patients). These findings raise questions of whether the effects of ADAs may evolve over time and, if so, for what reason; they also further support our previous conclusion

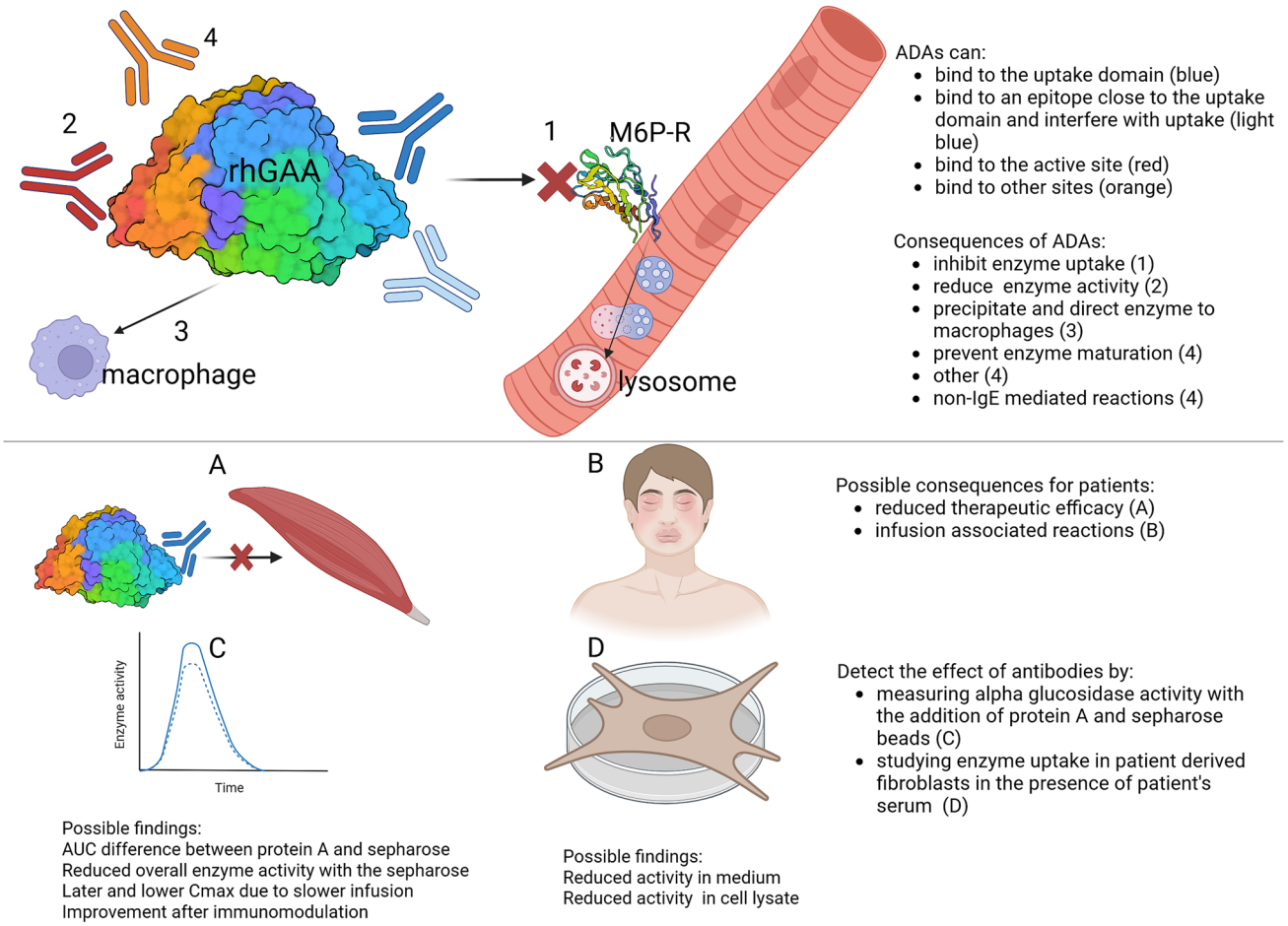


Fig. 5 Mechanisms of action of anti-drug antibodies (ADAs), possible consequences on the bioavailability of recombinant human acid α-glucosidase (rhGAA) and clinical consequences. Findings of this study were combined with previously published hypotheses, such as the prevention of enzyme maturation [28]. *AUC* area under the curve, C_{max} peak activity. Figure created in BioRender.com

that high antibody titers per se are insufficient to predict potential deleterious effects of antibodies [17]. Moreover, even among patients with high ADA titers, there was considerable heterogeneity in the observed effects of antibodies, as seen throughout the study and summarized in the last column of Table 1. The heterogeneous effects of ADAs are likely attributable to epitope-specific differences, whether they divert enzyme uptake into Fc receptor-positive cells, such as macrophages, target the uptake domain, bind to the active or the catalytic site [13, 28], or trigger non-IgE–mediated infusion reactions [24], as summarized in Fig. 5.

Taken together, these four effects ultimately lead to a reduction of available rhGAA for uptake in tissues. In five patients, ADAs bound 11–52% of infused rhGAA, as identified with the PK curves. This means that, after an infusion of rhGAA 40 mg/kg, 4.4–20.8 mg of rhGAA were captured, leaving 35.6–19.2 mg/kg available for tissue uptake, confirming previous findings of our group and highlighting the importance of high rhGAA doses [3, 8, 15]. Moreover, in four patients, ADAs reduced uptake in fibroblasts by 29–53% of total rhGAA, meaning that only 18.8–28.4 mg/kg of a theoretical 40-mg/kg dose of rhGAA would be available for uptake. If the patients had received the licensed dose of 20 mg/kg/every other week, hardly any rhGAA would be available for tissue uptake. Additionally, in a few patients, we found an overall lower enzyme activity in the sepharose assay and/or the medium, revealing that, also for them, less ERT was available for uptake, although it was not possible to quantify the amount. All these findings highlight the importance of high doses of ERT, which are necessary to counteract the effect of antibodies. The fourth consequence of ADAs was the development of IARs, which are managed by administering premedication and slowing the infusion. In terms of infusion schedule, it is important to note that, in comparison to other countries [29] and to the Summary of Product of Characteristics [30], the standard infusion

protocol in the Netherlands adopts a higher final infusion rate (15 mg/kg/h vs 7 mg/kg/h). In this study, slower infusion rates, as part of IARs management, were associated with a lower C_{max}, but not a lower AUC. It has been hypothesized that a shorter and faster infusion creates a stronger driving force of ERT to the tissues [31]. If C_{max} is, indeed, the driving force for getting ERT into the muscle, then the slower infusions would further reduce the bioavailability of rhGAA. However, the experiments conducted in the present study are not adequate to test this hypothesis.

Finally, this study confirms that immunomodulation, if performed correctly, eliminates ADAs and thereby their effects on the bioavailability of rhGAA. Indeed, in the two patients reported, immunomodulation with long-term RTX led to the disappearance of rhGAA precipitation after infusion and of uptake inhibition. In addition, after the resolution of IARs, the infusion schemes could be normalized and premedication suspended. Shorter infusion schemes are also associated with a better quality of life for patients and their families.

This study has several limitations. Although 15 PK curves represent a substantial group for a rare disease, the resulting subgroups remain small and statistically underpowered. Data were collected over a 15-year period, during which infusion timepoints varied slightly. These differences may influence AUC calculations and comparison on a group level, although it does not affect within-patient comparisons, as the sepharose assay was used as control. In contrast, C_{max} values remained consistent over time. Finally, uptake studies in fibroblasts were performed in only one of the three patients with a titer of 1:31,250.

To the best of our knowledge, this represents one of the largest available studies on the effects of anti-drug antibodies in a lysosomal storage disorder treated with enzyme replacement therapy. We believe that the principles and observations described here may be applicable to other lysosomal storage disorders also treated with ERT.

We hereby reported the effects of antibodies on the availability of rhGAA by measuring enzyme activity with the addition of protein A, which allowed us to quantify the amount of antibody-bound-rhGAA; however, such method does not provide information on the (in-)activity of the enzyme which is antibody-bound. More insight into the effects of antibodies on rhGAA could be provided by using LC-MS/MS and by expanding knowledge on the epitopes to which antibodies bind [32].

5 Conclusions

Our findings highlight the heterogeneous effects of high antibody titers on alglucosidase alfa bioavailability. We found that only high antibody titers, most commonly ≥ 1:156,250, had a measurable effect on the bioavailability of rhGAA and may lead to the capture of > 50% of enzyme in plasma during ERT infusion and a similar reduction in enzyme uptake in fibroblasts. It also shows that there is variability among patients in terms of effects of ADAs. Where available, functional assays, including protein A-based enzyme activity measurements and fibroblast uptake studies, can provide valuable additional information on the impact of antibodies on enzyme replacement therapy. Adequate immunomodulation reduces antibody titers, but regular antibody measurements remain mandatory thereafter. In case of renewed titer increase, additional immunomodulation may be considered.

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Declarations

Conflict of Interest A.T. v.d.P., J.M.P. v.d.H. and N.A.M.E v.d.B received funding for research, clinical trials, and/or as advisor from various industries working on ERT and next-generation therapies in the field of Pompe disease under agreements with Erasmus MC University Medical Center. W.W.M.P. is co-founder and unpaid Chief Scientific Officer of LentiCure, a company developing gene therapy for lysosomal disorders including Pompe disease. E.H.J. and M.H.W. received funding for clinical trial and contract work from Spark Therapeutics and Amicus Therapeutics. M.C.F, T.P., I.B., D.L., and S.G. declare no competing interests.

Ethical Approval Patients with Pompe disease followed at the Center for Lysosomal and Metabolic Diseases, Erasmus University Medical Center in Rotterdam, the Netherlands are enrolled in the prospective protocol MEC-2007-103, approved by the Medical Ethical Committee of Erasmus Medical Center.

Data Availability Statement All original data are present in the manuscript. Further enquiries can be addressed to the corresponding author.

Patient Consent Statement Patients and/or their parents or legal guardians provide written consent to participate in the study.

Author Contributions M.C.F participated in planning the project and design of experiments, collected data, analyzed and interpreted data and wrote the draft of the manuscript. A.T.P. and J.M.P.H. conceived

the project, designed experiments, interpreted data, participated in discussions and edited several versions of this manuscript. I.B. and T.P. contributed to PK analysis, results interpretation, improvement of figure quality and reviewed several versions of the paper. D.L., S.G., W.W.M.P. and N.A.M.E. v.d.B. participated in data interpretation and reviewed several drafts of the manuscript. E.H.J and M.H.W. supervised enzyme activity measurement and ELISA assay, analyzed and interpreted data and contributed to the manuscript preparation.

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