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Acute lymphoblastic leukaemia patients treated with PEGasparaginase develop antibodies to PEG and the succinate linker

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Summary

Polyethylene glycol (PEG) conjugated asparaginase (PEGasparaginase) is essential for treatment of paediatric acute lymphoblastic leukaemia. We developed an assay identifying antibodies against the PEG-moiety, the linker and the drug itself in patients experiencing hypersensitivity reactions to PEGasparaginase. Eighteen patients treated according to the DCOG ALL-11 protocol, with a neutralizing hypersensitivity reaction to PEGasparaginase to the first PEGasparaginase doses in induction (12 patients) or during intensification after interruption of several months (6 patients) were included. ELISA was used to measure antibodies, coating with the succinimidyl succinate linker conjugated to BSA, PEGfilgrastim and *Escherichia coli* asparaginase, and using hydrolysed PEGasparaginase and $mPEG_{5,000}$ for competition. Anti-PEG antibodies were detected in all patients (IgG 100%; IgM 67%) of whom 39% had anti-PEG antibodies exclusively. Pre-existing anti-PEG antibodies were also detected in patients who not previously received a PEGylated therapeutic (58% IgG; 21% IgM). Antibodies against the SS-linker were predominantly detected during induction (50% IgG; 42% IgM). Anti-asparaginase antibodies were detected in only 11% during induction but 94% during intensification. In conclusion, anti-PEG and anti-SS-linker antibodies predominantly play a role in the immunogenic response to PEGasparaginase during induction. Thus, switching to native *E. coli* asparaginase would be an option for adequate asparaginase treatment.

Keywords: acute lymphoblastic leukemia, PEGasparaginase, antibodies.

Asparaginase treatment is essential for childhood acute lymphoblastic leukaemia (ALL) treatment. The drug depletes extracellular asparagine, an essential amino acid for leukemic cells, selectively killing these cells (Muller & Boos, 1998). Because asparaginase is derived from bacteria, patients can develop antibodies to the non-human epitopes, neutralizing the drug completely. Neutralizing reactions can present with or without symptoms of an allergy, the latter being called silent inactivation (Oettgen *et al*, 1970; Peterson *et al*, 1971; Killander *et al*, 1976; Tong *et al*, 2014). These reactions mainly occur after an interruption of asparaginase treatment, during which anti-asparaginase antibody levels increase (Tong, *et al*, 2014). Three forms of asparaginase are clinically available which are derived from either *Escherichia coli* or *Erwinia chrysanthemi*. By conjugating native *E. coli* asparaginase with polyethylene glycol (PEG), the drug is less immunogenic (Abuchowski *et al*, 1977a; Abuchowski *et al*, 1977b; Knop *et al*, 2010). Therefore, PEGylated *E.coli* asparaginase (PEGasparaginase) is used for the treatment of paediatric ALL (Amylon *et al*, 1999; Rizzari *et al*, 2001; Silverman *et al*, 2001; Duval *et al*, 2002; Pession *et al*, 2005; Moghrabi *et al*, 2007). In case of a hypersensitivity reaction, patients have to switch from PEGasparaginase to asparaginase derived from *Erwinia chrysanthemi* bacteria for adequate treatment (Pieters *et al*, 2011).

PEGasparaginase consists of *E. coli* derived asparaginase, a 34·5 kDa tetramer to which 69–82 molecules of mPEG chains are conjugated using a succinimidyl succinate linker (SS-linker) on the ε-amino groups of lysine residues of the protein. Although PEGylation reduces the immunogenicity of

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asparaginase, neutralizing hypersensitivity reactions still occur (Tong *et al*, 2014). Surprisingly, these reactions seem to shift to the first PEGasparaginase doses in induction and not necessarily during the intensification course after an interruption of treatment, as seen in our patient cohort. This leads to the question whether antibodies to the PEG moiety or even the SS-linker may cause these reactions.

Although PEGylation decreases the immunogenicity of biotherapeutics, it has been reported that repeated administration of PEGylated therapeutics can induce anti-PEG antibodies associated with hypersensitivity reactions and rapid clearance Armstrong *et al*, 2007; Verhoef *et al*, 2014). Anti-PEG antibodies have been reported in patients treated with PEG conjugated uricase and PEGinesatide, erythropoietin that is covalently attached to PEG and withdrawn from the market after severe hypersensitivity reactions (Hershfield *et al*, 2014). Also in ALL-patients treated with PEGasparaginase, the formation of anti-PEG antibodies has been associated with rapid clearance of the drug (Armstrong *et al*, 2007).

We hypothesize that reactions to PEGasparaginase may be partly triggered by anti-PEG antibodies. Secondly, we hypothesize that the SS-linker can expose a neo-antigen to which antibodies can be formed. The linker contains an ester group which has limited stability at neutral pH in vitro and is subject to hydrolysis by endogenous esterases in vivo (Carter & Meyerhoff, 1985) The exposed succinate group may function as an hapten, enhancing immunogenicity (Carter & Meyerhoff, 1985; Turecek *et al*, 2016).

Because of the lack of proper developed anti-PEG antibody assays, especially lacking proper controls, the aim of this study was to develop a sensitive and specific assay to detect possible antibodies to both PEG and the SS-linker. For this we identified patients who had a neutralizing allergy to or silent inactivation of PEGasparaginase during the first administrations (induction phase) or after an interruption of PEGasparaginase treatment (intensification phase).

Methods

Patients and treatment protocol

Eighteen children with ALL treated according to the Dutch Childhood Oncology Group (DCOG) ALL-11 protocol between April 2012 and December 2016, who had developed a neutralizing hypersensitivity reaction (silent inactivation or allergy) to PEGasparaginase, were selected for the development of the assay. The DCOG ALL-11 protocol (Dutch Trial Register: NTR3379), including use of patient material, was approved by the Institutional Review Board and informed consent was obtained from patients >12 years old, parents or guardians in accordance with the declaration of Helsinki.

According to the DCOG ALL-11 protocol, all patients started with the induction phase containing prednisolone, vincristine daunorubicin, and PEGasparaginase (1500 IU/m^2 i.v. administered at day 12, 26 and 40). After induction, patients were stratified in a standard, medium or high risk group. In this study, all patients with a hypersensitivity reaction after induction were treated according to the medium risk group. After induction, two consolidation courses were given leading to an asparaginase-free interval of approximately 12 weeks. Thereafter, medium risk patients were treated with 14 PEGasparaginase doses, individualized based on asparaginase activity levels, in the intensification phase. If a hypersensitivity reaction occurred, patients were switched to *Erwinia* asparaginase.

To study the reactions specifically observed during the first PEGasparaginase doses, 12 patients with a neutralizing hypersensitivity reaction during the induction phase and 6 patients with a reaction during the intensification phase were selected (Table I). The antibodies were quantified in serum obtained within 2 weeks after the reaction. Allergies were graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 4.03.

Asparaginase activity

Whole blood was taken prior to each PEGasparaginase administration and centrifuged at 800 ***g*** for 10 min at room temperature (RT). Samples were stored at −20°C. PEGasparaginase activity levels were measured after thawing using the L-aspartic β-hydroxamate (AHA) assay as described earlier (Lanvers *et al*, 2002). Inactivation of PEGasparaginase is defined as PEGasparaginase activity level <100 IU/l at day 7 ± 1 after administration and/or a trough PEGasparaginase activity level (14 ± 1 days after a dose) below the limit of detection (<10 IU/l).

Size-exclusion chromatography of PEGasparaginase

The hydrolysis of $mPEG_{5,000}$ from PEGasparaginase (Oncaspar®, Shire, Amsterdam, the Netherlands) was investigated by size-exclusion chromatography. PEGasparaginase and native *E. coli* asparaginase (Paronal®, Medac, Wedel, Germany) were diluted to 100 IU/ml in 0.1 mol/l sodium bicarbonate buffer pH9·5 (Sigma Aldrich Zwijndrecht, the Netherlands) or PBS pH 7·4 (Fisher BioReagents, Landsmeer, the Netherlands) at RT for 30 h to investigate the dissociation at different pH. Size-exclusion chromatography was performed on a Waters 2695-Separations Module connected to a Waters 2414-Refractive Index Detector and a Waters 2487 Dual λ-Absorbance Detector (Waters Corporation, Milford, MA, USA) to which a PL-Aquagel-OH mixed 8 μm column (Agilent Technologies, Santa Clara, USA) was attached. Protein concentration was determined by UV-absorbance at 280 and 210 nm, the latter to correct for background. PEG polymer concentration was determined by the refractive index detector. Samples were incubated in one of the above buffers, and, over a 30 h time period at RT, injected at 1 ml/min flowrate. The use of size-exclusion chromatography allowed to separate the PEG from asparaginase and determine the concentration of both components over time as a results of hydrolysis.

Table I. Overview reactions and relative antibody titers.

				IgG				IgM			
Patient number	Type of reaction (grade)	Treatment phase	Dose number	Dilution PEGasp	Dilution Native *Escherichia coli* asp	Dilution PEG-filgrastim	Dilution BSA-SS	Dilution PEGasp	Dilution Native *Escherichia coli* asp	Dilution PEG-filgrastim	Dilution BSA-SS
1	Allergy (2)	Induction	3rd	16×	Negative	16×	2×	2×	Negative	1×	2×
2	Allergy (2)	Induction	3rd	128×	Negative	512×	1×	4×	Negative	4×	1×
3	Allergy (3)	Induction	1st	32×	Negative	128×	4×	4×	Negative	2×	4×
4	Allergy (2)	Induction	3rd	2×	Negative	8×	Negative	Negative	Negative	Negative	Negative
5	Allergy (3)	Induction	3rd	256×	Negative	1024×	2×	128×	Negative	128×	1×
6	Allergy (2)	Induction	1st	4×	1×	32×	2×	Negative	Negative	1×	2×
7	SI	Induction	3rd	64×	2×	512×	1×	2×	Negative	1×	Negative
8	SI	Induction	1st	Negative	Negative	8×	Negative	Negative	Negative	1×	Negative
9	SI	Induction	2nd	8×	Negative	8×	Negative	1×	Negative	Negative	Negative
10	SI	Induction	2nd	4×	Negative	8×	Negative	1×	Negative	1×	Negative
11	SI	Induction	3rd	16×	Negative	64×	Negative	Negative	Negative	Negative	Negative
12	SI	Induction	2nd	4×	Negative	16×	Negative	1×	Negative	1×	Negative
13	Allergy (3)	Intensification	2nd	256×	8×	512×	Negative	2×	Negative	2×	Negative
14	Allergy (2)	Intensification	2nd	256×	64×	1024×	1×	16×	1×	32×	1×
15	Allergy (2)	Intensification	2nd	64×	4×	512×	Negative	1×	Negative	2×	Negative
16	SI	Intensification	1st	1×	Negative	8×	Negative	Negative	Negative	Negative	Negative
17	SI	Intensification	1st	16×	2×	32×	Negative	8×	Negative	Negative	Negative
18	SI	Intensification	1st	32×	8×	64×	Negative	64×	4×	Negative	Negative

The minimal required dilutions to avoid background signal: Anti-PEGasparaginase IgG 1000×; IgM 2000×; native *E. coli* asparaginase IgG 1000×; IgM 2000×; PEG-filgrastim IgG 75×, IgM 75×; BSA-SS IgG 75×; IgM 75×. As these dilutions were different for the different antibodies, relative titers were calculated to compare the antibody titers. For this, the absolute titers measured were divided by the corresponding minimal required dilutions. For example, a relative titer of 16× means that the titer of that sample was 16 times higher than the negative samples. PEGasp, PEGasparaginase; asp, asparaginase; BSA-SS, bovine serum albumine – succinimidyl succinate; SI, silent inactivation.

Synthesis of the PEG BSA conjugate

BSA (Sigma Aldrich, Zwijndrecht, the Netherlands) was conjugated to succinic anhydride (BSA-SS) (Sigma-Aldrich, Zwijndrecht, the Netherlands) or n-ethyl maleimide (BSA-MAL) (Sigma-Aldrich, Zwijndrecht, the Netherlands) at a molar ratio of 1:10 in 0.1 mol/l sodium carbonate pH8·5, to model the exposed linker upon PEG hydrolysis. The pH was kept constant by adding 0.1 mol/l sodium hydroxide to the reaction. Upon conjugation, both solutions were dialyzed against 0.1 mol/l sodium carbonate pH8·5 and subsequently against PBS at 4°C. Protein concentration was determined by the Pierce™ BCA Protein Assay (Thermo Fisher Scientific, Breda, the Netherlands). Samples were stored at −20°C.

Anti-drug antibody determination

To investigate the specificity of antibodies against asparaginase, PEG and the SS-linker, an enzyme-linked immunosorbent assay (ELISA) was developed coating medium binding Costar® 96-well ELISA plates (Corning, Amsterdam, the Netherlands) with 0·4 IU/l PEGasparaginase, 1·4 IU/l native *E. coli* asparaginase, or 1 μg/ml of PEGfilgrastim (Neulasta, Amgen), filgrastim (Neupogen, Amgen), BSA or BSA-SS in PBS overnight. PEGfilgrastim, a 19 kDa protein to which a 20 kDa PEG is coupled by aldehyde chemistry using selective N-terminal amine conjugation, was used to investigate anti-PEG antibodies. Patient sera were screened for anti-filgrastim and anti-BSA antibodies to rule out false positive results. Plates were blocked with 2–5% BSA dissolved in PBS for a minimum of 2 h at room temperature. Patient sera were incubated for 2 h in block buffer. Plates were washed 5 times with 0·05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate CHAPS (Merck Chemicals, Amsterdam, the Netherlands) in PBS. IgG and IgM were detected by rabbit polyclonal anti-IgG or anti-IgM (Abcam, Cambridge, UK).

Patients were positive for antibodies if binding exceeded the cut-off point calculated by absorbance value of sera obtained from patients before their first PEGasparaginase administration who did not experience a hypersensitivity reaction ($n = 26$) as well as sera obtained from healthy donors ($n = 11$) (Mini Donor Dienst, UMCU, the Netherlands). The cut-off point for PEGfilgrastim and PEGasparaginase was determined in the presence of 0·1% $mPEG_{5,000}$ to exclude binding of possible pre-existing anti-PEG antibodies, which have been reported by others (Garay *et al*, 2012).

Specificity of antibodies was determined by competition with different concentrations of *E. coli* asparaginase, $mPEG_{5,000}$, or hydrolysed PEGasparaginase. For this, PEGasparaginase was diluted to 375 IU/ml in a 0.1 mol/l sodium carbonate buffer pH 9·8 and stored for 20 h at 37°C. Samples were concentrated by centrifugation in Vivaspin®20 tubes (GE-Healthcare, the Netherlands) and diluted in PBS. The pH was adjusted to pH7·4 by dropwise addition of 1 mol/l HCl.

To exclude any nonspecific competition of $mPEG_{5,000}$, its competitive properties were investigated in a varicella zoster virus (VZV) ELISA, as all patients are expected to be positive for VZV-antibodies. A polyclonal TransChromo bovine anti-PEG IgG antibody (Bristol-Myer Squibb, USA) was provided by Bristol-Myers Squibb to serve as a positive control.

Statistics

SPSS Statistics (IBM Corp, Armonk, New York, USA) version 21.0 and GraphPad Prism (GraphPad Software, Inc, La Jolla, USA) version 5.01 for Windows were used for statistical analyses. Cut-offs are defined as the mean plus one standard deviation. To compare the titers of different antibodies, relative titers were calculated by dividing the patient titer by the corresponding minimal required dilution. Relative titers were plotted with boxplots, with the 25, 50 and 75th percentiles in the boxes and the ranges indicated by whiskers. Dose response curves for the competition analyses were fitted using nonlinear regression.

RESULTS

Table I shows the 18 patients included as described in the Methods section. Of those, nine patients had silent inactivation of PEGasparaginase (6 patients during induction, 3 during intensification); the other patients had an allergy. Asparaginase activity levels measured after this allergic reaction were below the limit of quantification in all patients. Two patients had an allergic reaction against the first dose. Thus, only a small amount of PEGasparaginase was administered.

Antibodies against asparaginase and PEG

Presence of anti-PEGasparaginase antibodies, which could be directed to any epitope in the molecule, was investigated (Fig 1, Table I). Anti-PEGasparaginase IgG was detected in 92% (11/12) and 100% of the patients in respectively the induction and intensification phase; anti-PEGasparaginase IgM in 67% (8/12) and 83% (5/6). Antibody titers were higher for IgG than for IgM.

Specificity against asparaginase or PEG was investigated by coating plates with native *E. coli* asparaginase or PEGfilgrastim (Table II). In patients with a hypersensitivity reaction during induction, anti-asparaginase IgG was detected in 17% (2/12) and IgM in none of the patients. In contrast, these antibodies were detected in 83% (5/6) and 33% (2/6) of the patients with a reaction during intensification (Fig 1B, Tables I and II). All patients were positive for anti-PEG IgG antibodies, whereas 75% (9/12) and 50% (3/6) of patients were positive for anti-PEG IgM in the induction and intensification phase, respectively (Fig 1C, Tables I and II).

All patients were negative for anti-filgrastim IgG and IgM. Anti-PEG titers were around 100× higher for anti-PEG IgG than for anti-PEG IgM. Patients with undetectable

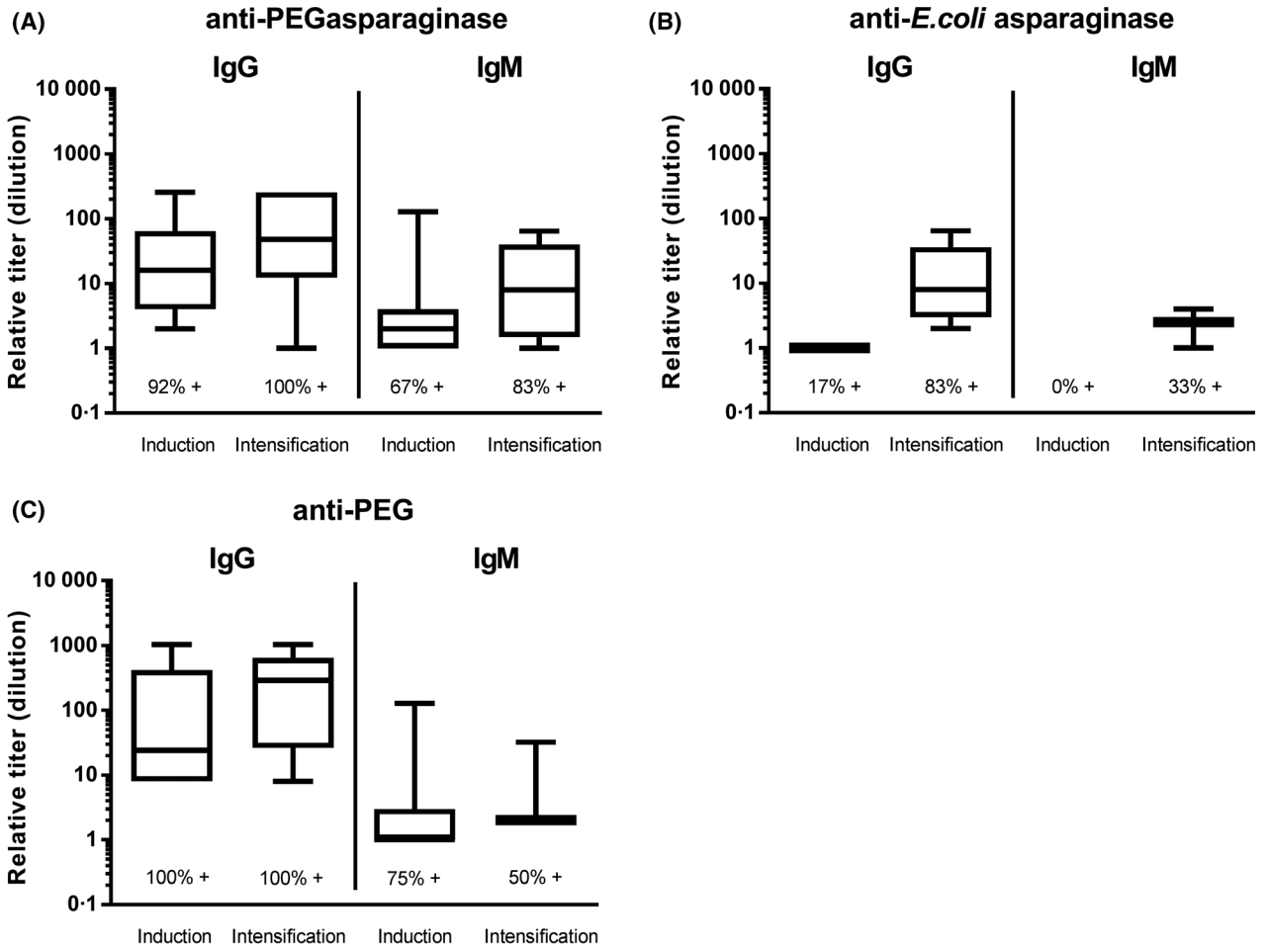

Fig 1. Shows the titers relative to the minimal required dilution per epitope for IgG and IgM in patients with a hypersensitivity reaction during the induction and intensification. Each graph also show the percentage of patients positive for the antibodies during induction (n = 12) and intensification (n = 6). The relative titers were obtained by dividing the titers measured by the corresponding minimal required dilutions. (A) Shows the relative titers of anti-PEGasparaginase antibodies. (B) Shows the relative titers of anti-native *Escherichia coli* asparaginase antibodies. (C) Shows the relative titers of anti-PEG antibodies. Minimal required dilutions (MRD): Anti-PEGasparaginase IgG 1000×; IgM 2000×; native *E. coli* asparaginase IgG 1000×; IgM 2000×; PEG-filgrastim IgG 75×, IgM 75×.

PEGasparaginase levels in combination with an allergic reaction had higher anti-PEG IgG titers than patients with silent inactivation. Although the anti-PEG titers were lower in the nine patients with silent inactivation, 6 out of these 9 patients were exclusively positive for anti-PEG antibodies (Table I). Specificity towards PEG was confirmed by $mPEG_{5,000}$ competition and validated by a polyclonal anti-PEG IgG antibody (Fig 2A) (Dong *et al*, 2015). Specificity of $mPEG_{5,000}$ to compete solely with anti-PEG antibodies was confirmed by its non-competitive properties to anti-VZV antibodies (Fig 2D).

Pre-existing anti-PEG IgG antibodies were detected in 58% (14/24) and anti-PEG IgM in 21% (5/24) patients (Fig 2C). These patients did not experience any hypersensitivity reaction to or neutralization of PEGasparaginase during asparaginase treatment.

Antibodies against the SS-linker

We found that PEG is rapidly hydrolysed from PEGasparaginase when incubated in sodium bicarbonate pH9·5 (Fig 3A).

Table II. Antibodies against asparaginase, PEG and the SS-linker in patients with a reaction to PEGasparaginase during induction or intensification.

Type of antibodies	Induction n = 12		Intensification n = 6	
	IgG (%)	IgM (%)	IgG (%)	IgM (%)
Anti-PEGasparaginase antibodies	92	67	100	83
Anti-Asparaginase antibodies	17	0	83	33
Anti-PEG antibodies	100	75	100	50
Anti-SS-linker antibodies	50	42	17	17

Specificity of anti-PEGasparaginase antibodies towards the SS-linker was determined by coating plates with BSA-SS. Anti-BSA-SS IgG was detected in 50% (6/12) and 17% (1/6) during the induction and intensification phase; anti-linker IgM in 42% (5/12) and 17% (1/6) (Fig 3B, Tables I and II). No antibodies were found against unmodified BSA or the unrelated BSA-MAL linker (Fig 3C). Competition by hydrolysed PEGasparaginase inhibited IgG and IgM binding towards BSA-SS in a dose-dependent manner (Fig 3D).

DISCUSSION

In this study we assessed the specificity of anti-drug antibodies in paediatric ALL patients treated with PEGasparaginase who had a neutralizing hypersensitivity reaction (silent inactivation or allergy) to the drug during the induction or intensification phase (Tong *et al*, 2014; van der Sluis *et al*, 2018). The patients were selected, though randomly, and the conclusions stated below should be confirmed by larger patient samples.

Patients with a hypersensitivity reaction to PEGasparaginase developed IgG and IgM antibodies towards asparaginase, the PEG chain and the linker. The incidence of anti-*E. coli* asparaginase antibodies in our sample was lower during induction (IgG 17% (2/12); IgM 0%) than intensification (IgG: 83% (5/6); IgM: 33% (2/6)), indicating that asparaginase antibodies are mainly developed in an PEGasparaginase-free period and are primarily IgG. Patients with a reaction during induction mainly had anti-PEG (100% IgG

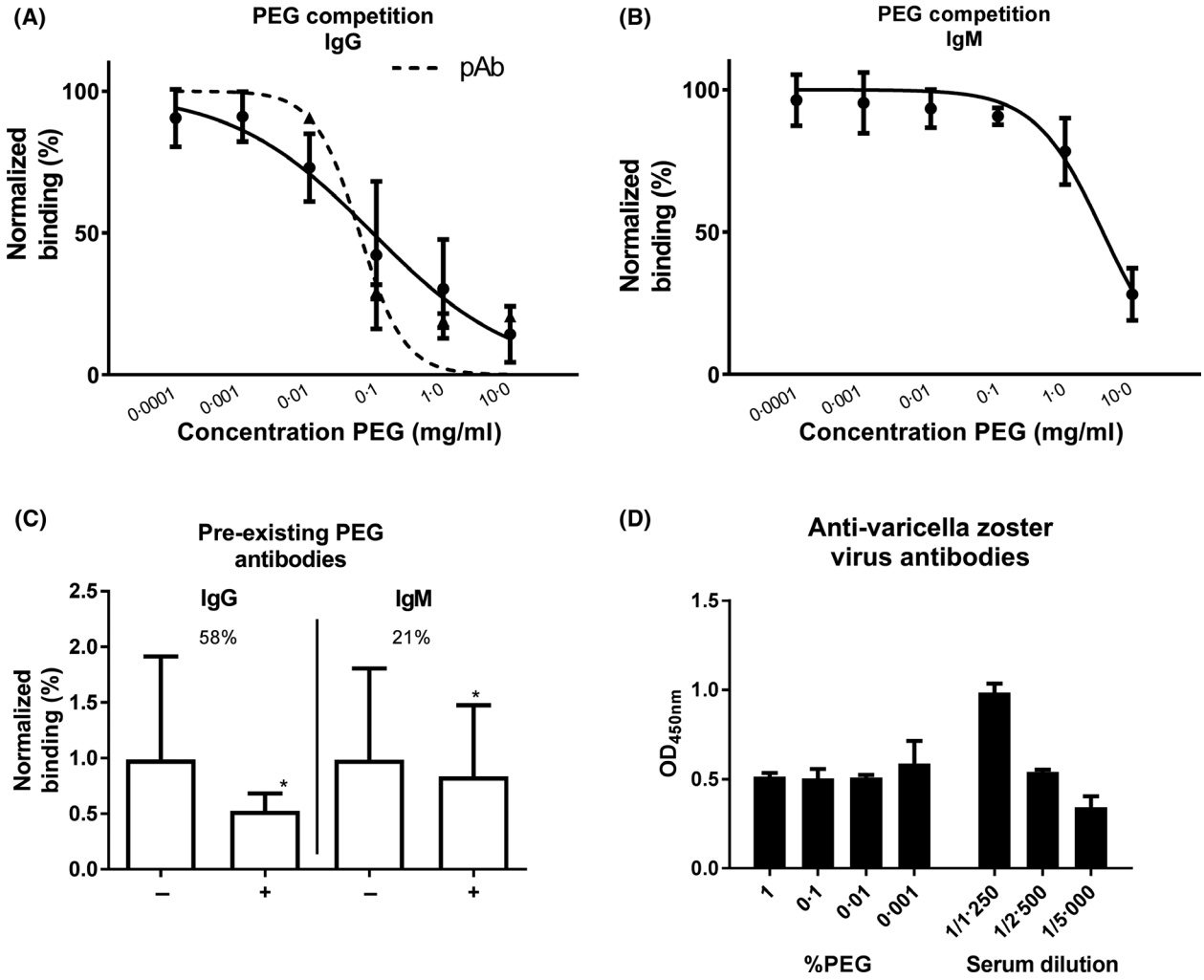

Fig 2. Shows the specificity of the anti-PEG antibodies in normalized dose-response curves. Gradually added $mPEG_{5,000}$ concentrations competed with the PEGfilgrastim, decreasing the extinction relative to the extinction without competition (%). (A) Shows the normalized dose-response curve for anti-PEG IgG with PEG-competition (n = 5) and the polyclonal anti-PEG IgG (pAb) as a positive control. (B) Shows the percentage of signal remaining after PEG competition for IgM (n = 6), which has an approximately 100× lower affinity for mPEG5k than IgG. (C) Shows normalized anti-PEG binding of patient samples obtained prior to the first PEGasparaginase dose, who did not have any symptoms of hypersensitivity or increased clearance of PEGasparaginase during treatment, in (+) or without (−) the presence of 0·1% $mPEG_{5,000}$. (D) Shows binding of healthy donor serum to varicella zoster virus antigen in the presence of different $mPEG_{5,000}$ concentrations. Also dilutions of sera are shown to indicate the doses-response relation in this assay.

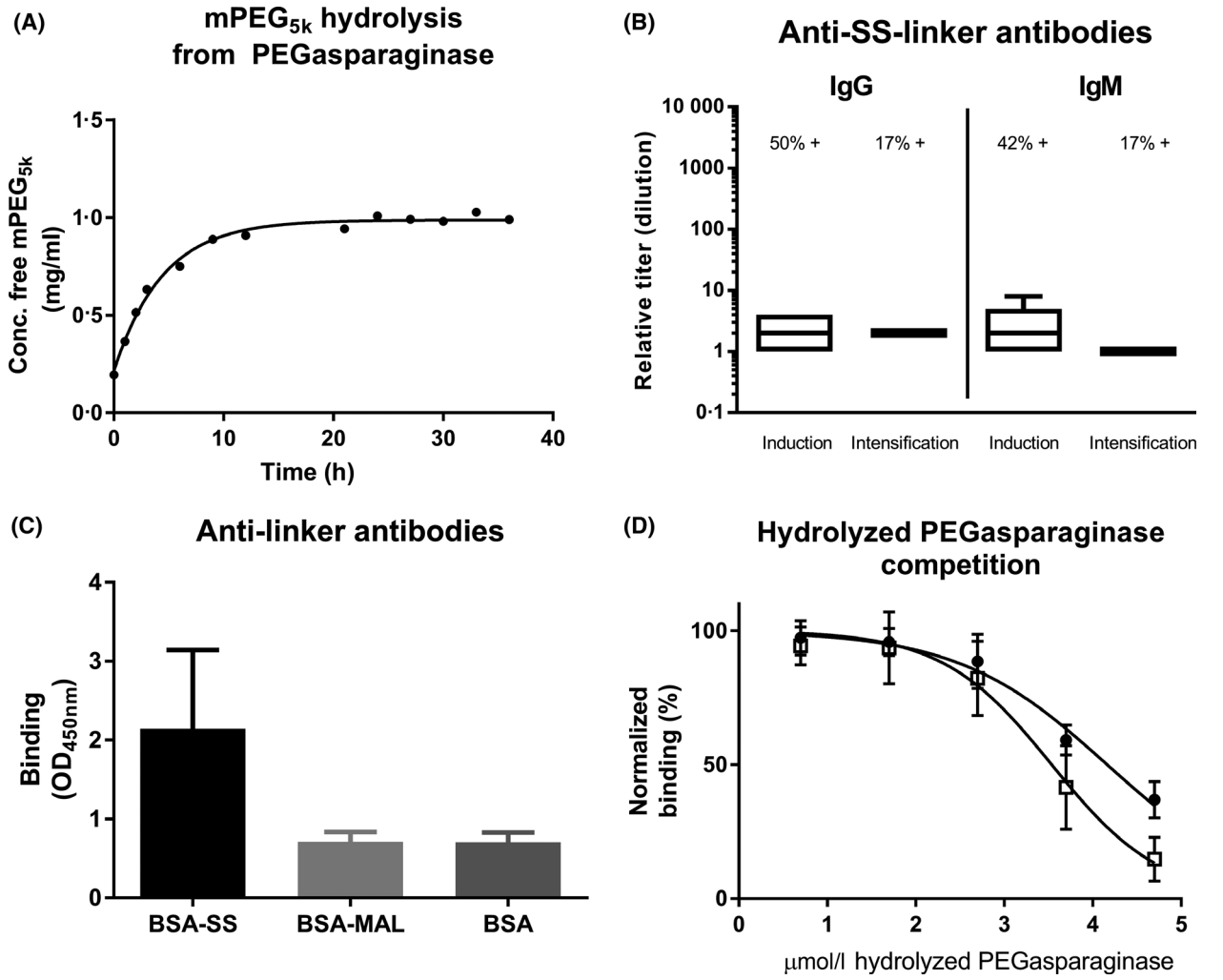

Fig 3. Shows the hydrolysis of PEGasparaginase and the detection of anti-succinate linker antibodies. (A) Shows the hydrolysis of $mPEG_{5,000}$ from PEGasparaginase at pH 9.5 and room temperature. After approximately 12 h, the concentration free $mPEG_{5k}$ stabilizes, showing complete hydrolysis of $mPEG_{5,000}$. (B) Shows the relative titers to the SS-linker conjugated to BSA. The relative titers were obtained by dividing the titers measured by the minimal required dilutions (75× for both IgG and IgM). Percentages indicate fraction of patients positive for anti-succinate antibodies. In (C) patients were screened for the n-ethyl maleimide group conjugated to BSA (random linker) and BSA. This figures shows that the antibodies were specific for the succinate group. (D) Shows IgG (●) and IgM (□) normalized dose-response curve of BSA-SS coated plates in presence of different concentrations of hydrolyzed PEGasparaginase.

and 75% IgM of 12 patients) and anti-succinate linker antibodies (50% IgG and 42% IgM of 12 patients). Thus, after a hypersensitivity reaction during the first PEGasparaginase administrations, patients might benefit from a switch to native *E. coli* asparaginase instead of *Erwinia* asparaginase for adequate treatment.

In contrast to *E. coli* asparaginase antibodies, the patients from our cohort were positive for anti-PEG IgG and IgM antibodies during hypersensitivity reactions both treatment phases. Mainly patients with an allergy had high anti-PEG antibody titers, suggesting that these antibodies may induce clinical symptoms of an allergy. Also, 39% (7/18) with a hypersensitivity reaction had exclusively anti-PEG antibodies, confirming the neutralizing capacity of these antibodies. Overall, IgM titers were low compared to anti-PEG IgG suggesting isotype class switching. Previous articles suggest that anti-PEG antibodies are predominantly induced through a T-cell independent manner (Ishida *et al*, 2007). Production of anti-drug antibodies that bypass T-cells are typically IgM or low-affinity IgG (Baker *et al*, 2010). Binding of these antibodies can form immune complexes that are recognized by Fc- or complement-receptors promoting uptake by antigen processing cells such as splenic marginal B-cells (Krishna & Nadler, 2016). The presence of pre-existing anti-PEG antibodies suggest a mechanism of memory B-cells. Although effective B-cell memory requires involvement of T_h-cells, there is growing evidence that T-cell independent type II antigens can form B-cell memory (Good-Jacobson & Tarlinton, 2012). T-cell independent type II antigens are associated with polysaccharides, bearing repetitive structures (Obukhanych & Nussenzweig, 2006). The repetitive

ethylene oxide (-CH_2-CH_2-O-) units of PEG may be recognized by the same mechanism as these T-cell independent type II antigens.

In line with our observations, Rau *et al*, 2017 reported lower anti-PEG IgM titers than IgG in patients with an allergic reaction to PEGcrisantaspase, a PEGylated form of recombinant *Erwinia* asparaginase using glutaric acid spacer as a linker, administered after a hypersensitivity reaction to PEGasparaginase. Whereas anti-PEG antibodies detected in animals are predominantly reported as anti-PEG IgM, human studies primarily show anti-PEG IgG (Verhoef *et al*, 2014; Yang & Lai, 2015).

In our study, 58% (14/24) of the patients without any reaction had pre-existing anti-PEG IgG, and 21% anti-PEG IgM. Although anti-PEG antibodies were already described in the healthy population (Armstrong *et al*, 2003), this study shows that the antibodies can be developed relatively early in life and may have been formed during previous exposure to PEG containing food or cosmetic products (Richter & Akerblom, 1984; Leger *et al*, 2001; Armstrong *et al*, 2003; Liu *et al*, 2011). The fact that also patients without neutralization had anti-PEG antibodies proves that these antibodies do not necessarily possess neutralizing characteristics. Why these antibodies result in a neutralizing hypersensitivity reaction in only part of the patients should be further investigated.

Our study is the first to report that antibodies can be formed against the succinate succinimidyl linker and provides clinical evidence that use of a cleavable linker that remains on the therapeutic protein upon hydrolysis can induce the formation of anti-drug antibodies. Although *in vivo* no such basic conditions are expected, the hydrolysis results are intended to show that the linker is hydrolysis prone. As there are several hydrolytic enzymes in the body present, we raise the hypothesis that these are responsible for hydrolyzing the PEG from the protein. Anti-SS-linker antibodies were predominantly found during induction. However, all patients positive for anti-SS-linker antibodies also had anti-PEG antibodies. We could, therefore, not identify if these anti-linker antibodies only bind to the linker or also induce an immunological response. The anti-PEG antibodies may also be directed towards both the linker and the PEG chain. Angiolillo et al. studied the presence of antibodies and occurrence of hypersensitivity reactions in patients treated with PEGasparaginase or Calaspargase pegol, a PEGylated *E. coli* asparaginase with a succinimidyl carbamate linker. Treatment with this type of asparaginase seems to result in a less rapid clearance and slightly less hypersensitivity reactions during induction (3% vs. 7%, although not significant) (Angiolillo *et al*, 2014). These findings may imply that PEGylation with the SS-linker results in a more immunogenic therapeutic than by the other linker.

Anti-PEGasparaginase antibodies are frequently measured by various study groups with ELISA using a standard carbonate buffer of pH 9·0–9·5. However, PEG hydrolyses from PEGasparaginase at pH9·5 (Fig 3A). Thus, by using this buffer only anti-*E. coli* asparaginase and anti-SS-linker antibodies can be measured. Therefore, we recommend to coat plates using a buffer of pH7·4 for PEGasparaginase antibody detection.

In conclusion, first, we identified that anti-PEG antibodies play a significant role in the neutralization of PEGasparaginase, mainly during the first doses in induction, since part of the patients are only positive for this type of antibodies. Second, anti-asparaginase antibodies are almost exclusively developed during hypersensitivity reactions after an asparaginase-free interval in our patient cohort. Thus, patients with a reaction during the first doses of PEGasparaginase theoretically could switch to the less expensive native *E. coli* asparaginase for adequate treatment, reserving *Erwinia* asparaginase as an extra alternative. Third, pre-existing anti-PEG antibodies are found in part of the patients with no clinical effect. Therefore, these antibodies not necessarily trigger a hypersensitivity reaction and screening of anti-PEG antibodies is not a good marker to estimate patient outcome. And fourth, the discovery of the SS-linker as a neo-antigen shows that PEGylation through an unstable linker remaining on the therapeutic protein upon hydrolysis can serve as a hapten. Future drug development efforts should therefore focus on stable PEG-linker chemistries.

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Conflict of interest

Authors Robin Q.H. Kloos, Enrico Mastrobattista, Wim Hennink, and Jan-Jaap Verhoef declare that they have no conflict of interest. Authors Rob Pieters and I.M. van der Sluis received research support and consultancy fees from Jazz Pharmaceuticals and Medac.

References

Abuchowski, A., McCoy, J.R., Palczuk, N.C., van Es, T. & Davis, F.F. (1977a) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. *Journal of Biological Chemistry*, **252**, 3582–3586.

Abuchowski, A., van Es, T., Palczuk, N.C. & Davis, F.F. (1977b) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. *Journal of Biological Chemistry*, **252**, 3578–3581.

Amylon, M.D., Shuster, J., Pullen, J., Berard, C., Link, M.P., Wharam, M., Katz, J., Yu, A., Laver, J., Ravindranath, Y., Kurtzberg, J., Desai, S.,

Camitta, B. & Murphy, S.B. (1999) Intensive high-dose asparaginase consolidation improves survival for pediatric patients with T cell acute lymphoblastic leukemia and advanced stage lymphoblastic lymphoma: a Pediatric Oncology Group study. *Leukemia*, **13**, 335–342.

Angiolillo, A.L., Schore, R.J., Devidas, M., Borowitz, M.J., Carroll, A.J., Gastier-Foster, J.M., Heerema, N.A., Keilani, T., Lane, A.R., Loh, M.L., Reaman, G.H., Adamson, P.C., Wood, B., Wood, C., Zheng, H.W., Raetz, E.A., Winick, N.J., Carroll, W.L. & Hunger, S.P. (2014) Pharmacokinetic and pharmacodynamic properties of calaspargase pegol Escherichia coli L-asparaginase in the treatment of patients with acute lymphoblastic Leukemia: results from Children's Oncology Group Study AALL07P4. *Journal of Clinical Oncology*, **32**, 3874–3882.

Armstrong, J.K., Leger, R., Wenby, R.B., Meiselman, H.J., Garratty, G. & Fisher, T.C. (2003) Occurrence of an antibody to poly(ethylene glycol) in normal donors. *Blood*, **102**, 556a–556a.

Armstrong, J.K., Hempel, G., Koling, S., Chan, L.S., Fisher, T., Meiselman, H.J. & Garratty, G. (2007) Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer*, **110**, 103–111.

Baker, M.P., Reynolds, H.M., Lumicisi, B. & Bryson, C.J. (2010) Immunogenicity of protein therapeutics: the key causes, consequences and challenges. *Self Nonself*, **1**, 314–322.

Carter, M.C. & Meyerhoff, M.E. (1985) Instability of succinyl ester linkages in O2'-monosuccinyl cyclic AMP-protein conjugates at neutral pH. *Journal of Immunological Methods*, **81**, 245–257.

Dong, H., Mora, J.R., Brockus, C., Chilewski, S.D., Dodge, R., Merrifield, C., Dickerson, W.M. & DeSilva, B. (2015) Development of a generic anti-PEG antibody assay using BioScale's acoustic membrane microparticle technology. *American Association of Pharmaceutical Scientists Journal*, **17**, 1511–1516.

Duval, M., Suciu, S., Ferster, A., Rialland, X., Nelken, B., Lutz, P., Benoit, Y., Robert, A., Manel, A.M., Vilmer, E., Otten, J. & Philippe, N. (2002) Comparison of Escherichia coli-asparaginase with Erwinia-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer-Children's Leukemia Group phase 3 trial. *Blood*, **99**, 2734–2739.

Garay, R.P., El-Gewely, R., Armstrong, J.K., Garratty, G. & Richette, P. (2012) Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. *Expert Opinion on Drug Delivery*, **9**, 1319–1323.

Good-Jacobson, K.L. & Tarlinton, D.M. (2012) Multiple routes to B-cell memory. *International Immunology*, **24**, 403–408.

Hershfield, M.S., Ganson, N.J., Kelly, S.J., Scarlett, E.L., Jaggers, D.A. & Sundy, J.S. (2014) Induced and pre-existing anti-polyethylene glycol antibody in a trial of every 3-week dosing of pegloticase for refractory gout, including in organ transplant recipients. *Arthritis Research & Therapy*, **16**, R63.

Ishida, T., Wang, X., Shimizu, T., Nawata, K. & Kiwada, H. (2007) PEGylated liposomes elicit an anti-PEG IgM response in a T cell-independent manner. *Journal of Controlled Release*, **122**, 349–355.

Killander, D., Dohlwitz, A., Engstedt, L., Franzen, S., Gahrton, G., Gullbring, B., Holm, G., Holmgren, A., Hoglund, S., Killander, A., Lockner, D., Mellstedt, H., Moe, P.J., Palmblad, J., Reizenstein, P., Skarberg, K.O., Swedberg, B., Uden, A.M., Wadman, B., Wide, L. & Ahstrom, L. (1976) Hypersensitive reactions and antibody formation during L-asparaginase treatment of children and adults with acute leukemia. *Cancer*, **37**, 220–228.

Knop, K., Hoogenboom, R., Fischer, D. & Schubert, U.S. (2010) Poly(ethylene glycol) in drug delivery: pros and cons as well as potential alternatives. *Angewandte Chemie (International ed. in English)*, **49**, 6288–6308.

Krishna, M. & Nadler, S.G. (2016) Immunogenicity to biotherapeutics – the role of anti-drug immune complexes. *Frontiers in Immunology*, **7**, 21.

Lanvers, C., Vieira Pinheiro, J.P., Hempel, G., Wuerthwein, G. & Boos, J. (2002) Analytical validation of a microplate reader-based method for the therapeutic drug monitoring of L-asparaginase in human serum. *Analytical Biochemistry*, **309**, 117–126.

Leger, R.M., Arndt, P., Garratty, G., Armstrong, J.K., Meiselman, H.J. & Fisher, T.C. (2001) Normal donor sera can contain antibodies to polyethylene glycol (PEG). *Transfusion*, **41**, 29s–30s.

Liu, Y., Reidler, H., Pan, J., Milunic, D., Qin, D., Chen, D., Vallejo, Y.R. & Yin, R. (2011) A double antigen bridging immunogenicity ELISA for the detection of antibodies to polyethylene glycol polymers. *Journal of Pharmacological and Toxicological Methods*, **64**, 238–245.

Moghrabi, A., Levy, D.E., Asselin, B., Barr, R., Clavell, L., Hurwitz, C., Samson, Y., Schorin, M., Dalton, V.K., Lipshultz, S.E., Neuberg, D.S., Gelber, R.D., Cohen, H.J., Sallan, S.E. & Silverman, L.B. (2007) Results of the Dana-Farber Cancer Institute ALL Consortium Protocol 95–01 for children with acute lymphoblastic leukemia. *Blood*, **109**, 896–904.

Muller, H.J. & Boos, J. (1998) Use of L-asparaginase in childhood ALL. *Critical Reviews in Oncology Hematology*, **28**, 97–113.

Obukhanych, T.V. & Nussenzweig, M.C. (2006) T-independent type II immune responses generate memory B cells. *The Journal of Experimental Medicine*, **203**, 305–310.

Oettgen, H.F., Stephenson, P.A., Schwartz, M.K., Leeper, R.D., Tallai, L., Tan, C.C., Clarkson, B.D., Golbey, R.B., Krakoff, I.H., Karnofsky, D.A., Murphy, M.L. & Burchenal, J.H. (1970) Toxicity of E. coli L-asparaginase in man. *Cancer*, **25**, 253–278.

Pession, A., Valsecchi, M.G., Masera, G., Kamps, W.A., Magyarosy, E., Rizzari, C., van Wering, E.R., Lo Nigro, L., van der Does, A., Locatelli, F., Basso, G. & Arico, M. (2005) Long-term results of a randomized trial on extended use of high dose L-asparaginase for standard risk childhood acute lymphoblastic leukemia. *Journal of Clinical Oncology*, **23**, 7161–7167.

Peterson, R.G., Handschumacher, R.E. & Mitchell, M.S. (1971) Immunological responses to L-asparaginase. *Journal of Clinical Investigation*, **50**, 1080–1090.

Pieters, R., Hunger, S.P., Boos, J., Rizzari, C., Silverman, L., Baruchel, A., Goekbuget, N., Schrappe, M. & Pui, C.H. (2011) L-asparaginase treatment in acute lymphoblastic leukemia: a focus on Erwinia asparaginase. *Cancer*, **117**, 238–249.

Rau, R.E., Dreyer, Z., Choi, M.R., Liang, W., Skowronski, R., Allamneni, K.P., Devidas, M., Raetz, E.A., Adamson, P.C., Blaney, S.M., Loh, M.L. & Hunger, S.P. (2017) Outcome of pediatric patients with acute lymphoblastic leukemia/lymphoblastic lymphoma with hypersensitivity to pegaspargase treated with PEGylated Erwinia asparaginase, pegcrisantaspase: a report from the Children's Oncology Group. *Pediatric Blood & Cancer*, **65**. https://doi.org/10.1002/pbc.26873.

Richter, A.W. & Akerblom, E. (1984) Polyethylene glycol reactive antibodies in man: titer distribution in allergic patients treated with monomethoxy polyethylene glycol modified allergens or placebo, and in healthy blood donors. *International Archives of Allergy and Applied Immunology*, **74**, 36–39.

Rizzari, C., Valsecchi, M.G., Arico, M., Conter, V., Testi, A., Barisone, E., Casale, F., Lo Nigro, L., Rondelli, R., Basso, G., Santoro, N., Masera, G.; Associazione Italiano Ematologia Oncologia Pediatrica. (2001) Effect of protracted high-dose L-asparaginase given as a second exposure in a Berlin-Frankfurt-Munster-based treatment: results of the randomized 9102 intermediate-risk childhood acute lymphoblastic leukemia study–a report from the Associazione Italiana Ematologia Oncologia Pediatrica. *Journal of Clinical Oncology*, **19**, 1297–1303.

Silverman, L.B., Gelber, R.D., Dalton, V.K., Asselin, B.L., Barr, R.D., Clavell, L.A., Hurwitz, C.A., Moghrabi, A., Samson, Y., Schorin, M.A., Arkin, S., Declerck, L., Cohen, H.J. & Sallan, S.E. (2001) Improved outcome for children with acute lymphoblastic leukemia: results of Dana-Farber Consortium Protocol 91–01. *Blood*, **97**, 1211–1218.

van der Sluis, I.M., de Groot-Kruseman, H., Te Loo, M., Tissing, W.J.E., van den Bos, C., Kaspers, G.J.L., Bierings, M., Kollen, W.J.W., Konig, T., Pichlmeier, U., Kuhnel, H.J. & Pieters, R. (2018) Efficacy and safety of recombinant E. coli asparaginase in children with previously untreated acute lymphoblastic leukemia: a randomized multicenter study of the Dutch Childhood Oncology Group. *Pediatric Blood and Cancer*, **65**, e27083.

Tong, W.H., Pieters, R., Kaspers, G.J., te Loo, D.M., Bierings, M.B., van den Bos, C., Kollen, W.J., Hop, W.C., Lanvers-Kaminsky, C., Relling, M.V., Tissing, W.J. & van der Sluis, I.M. (2014) A prospective study on drug

monitoring of PEGasparaginase and Erwinia asparaginase and asparaginase antibodies in pediatric acute lymphoblastic leukemia. *Blood*, **123**, 2026–2033.

Turecek, P.L., Bossard, M.J., Schoetens, F. & Ivens, I.A. (2016) PEGylation of biopharmaceuticals: a review of chemistry and nonclinical safety information of approved drugs. *Journal of Pharmaceutical Sciences*, **105**, 460–475.

Verhoef, J.J., Carpenter, J.F., Anchordoquy, T.J. & Schellekens, H. (2014) Potential induction of anti-PEG antibodies and complement activation toward PEGylated therapeutics. *Drug Discovery Today*, **19**, 1945–1952.

Yang, Q. & Lai, S.K. (2015) Anti-PEG immunity: emergence, characteristics, and unaddressed questions. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology*, **7**, 655–677.