

Aluminium from adjuvanted subcutaneous allergen immunotherapeutics in rats is mainly detected in bone

To the Editor,

Aluminium (Al) hydroxide (AH) is frequently used as adjuvant in subcutaneous allergen immunotherapy (SCIT) in Europe. With regard to the cumulative Al amount administered per year, SCIT constitutes an important source of Al in humans, and its safety is constantly questioned in the public. Toxicokinetics of Al after SC injection of those poorly soluble Al particles is unknown. We conducted a study in rats receiving a single SC injection (1 mL) of either plain AH adjuvant (pAH: Alhydrogel®, 1.25 mg Al/mL; *n* = 6 rats), each of two marketed AH-adjuvanted SCIT products at highest marketed strength with high Al content but different in adjuvant manufacture (P1: Alhydrogel®-adjuvanted mixed grass pollen allergen, 1 mg Al/mL; P2: in situ co-precipitated AH-adjuvanted mixed grass pollen allergen, 1.13 mg Al/mL; *n* = 6 rats each), or vehicle (saline; *n* = 3 rats). Al in plasma (during 80 days) and tissues (at day 80; whole humerus bone and right brain hemisphere) was determined by atomic absorption spectrometric (AAS) procedures (full Method section in Appendix S1).

Al plasma time courses after treatment did not exhibit profiles distinctive from that of the vehicle group (Figure 1); however, for P2, but not for P1 and pAH, a significant increase in Al plasma AUC_(0-80 d) vs vehicle (mean [SD]: 1431 [314] vs 909 [48] µg/L*d) was found.

Al concentration in bone was significantly increased in all groups (Figure 2; Table S2), again strongest after P2 (mean [SD]: 1.28 [0.26], 0.64 [0.12], 0.72 [0.10] and 0.40 [0.11] µg/g wet weight (ww) for P2, P1, pAH and vehicle, respectively). However, concentrations were far below the levels of toxicological concern (>10–15 µg/g in both rats and humans^{1,2}). A more visible increase in bone rather than plasma exposure is not surprising as in contrast to fast renal Al plasma clearance, elimination of Al from bone is very slow and skeleton is the major storage compartment for Al in the body of both rats and humans.³

No significant difference in brain Al concentration between any treatment and vehicle was observed (means: 0.07–0.29 vs 0.08 µg/g ww; Figure 2; full data see Table S2), which is consistent with observations of very small fractions of dose (<0.01%) retaining in brain.³ Since we measured the Al amount in a whole hemisphere, clusters due to focal accumulation as well as any undissolved Al particles potentially transported into the brain⁴ are fully captured. We conclude from our results that contribution of such particulate Al amounts in brain, if any, is negligible.

The findings in plasma and bone were corroborated by determinations at the injection site: a high Al release (65.9% of injected dose) was found for P2 in contrast to small dose fractions for plain Alhydrogel® and Alhydrogel®-adjuvanted product P1 (1.7 and 11.2%,

resp.; Figure S3 and Table S2). The highest absolute Al amount released after 80 days was 659.1 µg for P2, being about fivefold compared with that for P1 (126.6 µg). Frequency of persistent nodules at day 80 was higher in the groups receiving pAH (6/6 rats) and P1 (5/6) compared with P2 (3/6), showing a plausible inverse relation to the Al release from the injection site.

A positive linear relationship was found between Al amount released from the injection site until day 80 and Al plasma AUC_(0-80 d) ($y = 0.73x + 927$; $r = .79$) or bone Al concentration ($y = 0.00079x + 0.63$; $r = .70$), respectively (Figure S4). These correlations confirm that Al release was an indirect measure of the systemically available amount. However, we cannot exclude some overestimation of this amount as a fraction of insoluble AH might have been transported to the draining lymph node but not yet entered the system.

To our knowledge, this is the first time that Al from marketed SCIT products has been systemically detected. Increase in Al levels was mainly visible in bone, less in plasma, but not in brain. Furthermore, our data indicate that Al-adjuvanted SCIT products do not behave uniformly: we observed a remarkable difference in the degree of systemic Al availability at day 80 between two marketed AH-adjuvanted SCIT products, suggesting a higher rate of Al release from the co-precipitated product. This is most probably due to the physicochemical differences (ie chemical composition

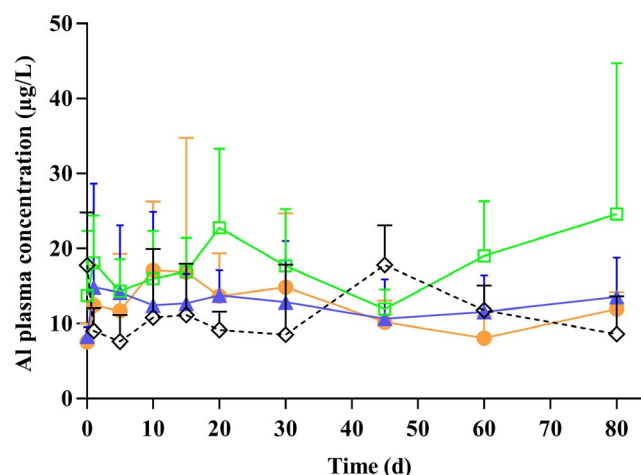


FIGURE 1 Mean (+SD) Al plasma concentration-time course in rats after single SC administration of plain AH adjuvant (circles; *n* = 6), adjuvanted SCIT products P1 (triangles; *n* = 6) or P2 (squares; *n* = 6), or vehicle (diamonds; dotted line; *n* = 3) [Colour figure can be viewed at wileyonlinelibrary.com]

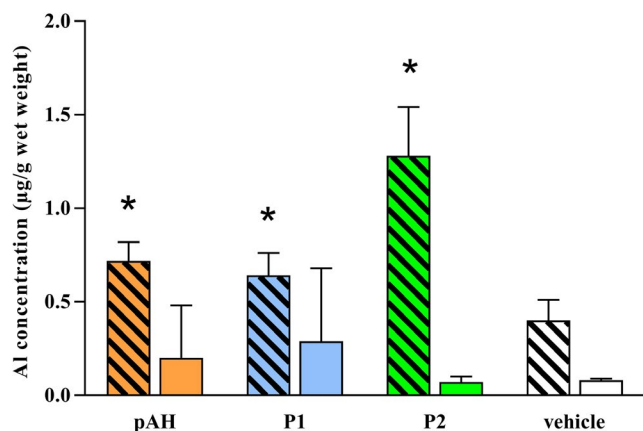


FIGURE 2 Mean (+SD) Al concentration in bone (striped bars) and brain (unfilled bars) of rats on day 80 after SC injection of plain AH adjuvant (pAH), adjuvanted SCIT products P1 or P2, or vehicle. * $P < .05$ (ANOVA compared with vehicle) [Colour figure can be viewed at wileyonlinelibrary.com]

and amorphous state) of co-precipitated AH adjuvants compared with preformed commercial AH adjuvants such as Alhydrogel®.⁵

However, once absorption is completed, dose-normalized cumulative extent of bone Al increase is expected to be comparable between products. Crude linear extrapolation of our injection site release data over time (including literature data⁶) suggests that complete absorption from Alhydrogel®-adjuvanted allergen preparations takes at least 350 days, whereas absorption from co-precipitated products might be completed much earlier (after ca. 120 days).

Our results cannot one-to-one be translated to humans. In relation to body weight, the doses applied to our rats (350 g) were 170 times higher compared with a 60 kg human adult. Considering an allometric scaling factor of 6.2 usually applied for dose conversion on mg/kg basis between rats and humans in pharmacology,⁷ this ratio is still 27. Thus, increase in bone Al concentration in a human adult after absorption of a SCIT Al dose of 659 µg (estimated Al release after P2 on day 80) is assumed 1/27 of that observed in our rats. Based on this calculation, we expect that after a single SCIT dose in humans, Al levels in bone, and even more valid in plasma and brain, will be indistinguishable from baseline levels. However, assuming full accumulation during a 3-year perennial SCIT applying 36 maintenance doses each containing up to 1250 µg Al in adults a cumulative bone Al increase of 1–2 µg/g wet weight is roughly extrapolated. Compared with an upper limit of normal bone Al content in healthy humans of <10 µg/g dry weight,^{1,8} this increase is considered substantial but without clinical relevance. For more precise predictions of Al exposure according to various treatment schedules, particularly in children, a physiology-based toxicokinetic (PBTK) model is desirable.⁹ For its establishment, the results of this study are considered essential.

CONFLICT OF INTEREST

Author Jennifer D. Oduro declares that she is employee at preclinics GmbH, a contract research organization that has received payment

for the conductance of the animal study. All other authors declare that they have no conflict of interest.

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SUPPORTING INFORMATION

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

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Allergenomics of the tick *Ixodes ricinus* reveals important α -Gal-carrying IgE-binding proteins in red meat allergy

To the Editor

Red meat allergy known as mammalian meat allergy, caused by IgE antibodies against galactose- α -1,3-galactose (α -Gal), is nowadays recognized worldwide, and strong associations with tick bites have been identified for different tick species and geographical locations.¹ Time relationship between tick exposure and increased IgE levels to α -Gal has further supported the strong evidence that tick bites are the primary cause of the IgE antibodies.² Ticks in all developmental stages can bite humans, and in the United States, high IgE levels to α -Gal following bites from larvae have been reported.³ While we have previously demonstrated the presence of α -Gal in the gut of the European tick *Ixodes ricinus*,⁴ α -Gal-containing proteins in tick saliva from the South American, Japanese and European ticks, *Amblyomma sculptum*,⁵ *Haemaphysalis longicornis*⁶ and *Hyalomma marginatum*,⁷ have recently been reported. However, the α -Gal content of the *I. ricinus* proteome has not been investigated yet. Here, we used allergenomics⁸ and shotgun proteomics approaches to identify IgE-binding α -Gal-carrying proteins in adult and larval stages and in saliva of *I. ricinus* ticks. Allergen-specific IgE antibody responses were assessed by ImmunoCAP (Thermo Fisher Scientific) among 32 Swedish and 18 US mammalian meat allergic patients to reveal differences in IgE reactivity between two geographical population groups. IgE- and α -Gal-binding capacities, as well as allergenicity of *I. ricinus* tick proteins, were evaluated (details on Methods and Patients' characteristics are presented in the Appendix S1).

Our data revealed that nearly all Swedish and American meat allergic patients had IgE responses against both the larval and adult stages of *I. ricinus*, and we noted that the four individuals IgE negative to *I. ricinus* had comparably low IgE levels to α -Gal (Table S1). Similar to previous reports, moderate-to-high correlations between IgE to α -Gal and adult ticks were seen in the two patient groups (Figure S1).⁴ Furthermore, the strong correlations between IgE reactivity to adult and larvae in both patient groups suggest that growth stages of *I. ricinus* ticks seem to be of less importance for the IgE recognition (Figure S1). In Western blot, IgE-reactive protein bands in the molecular range of 25-150 kDa in *I. ricinus* ticks were noted with comparable results for both tick stages using Swedish and American serum pools (Figure 1A,B, respectively). To investigate whether the observed IgE

reactivities were α -Gal-related, the pools were preincubated with the α -Gal-carrying glycoprotein bovine thyroglobulin prior to immunoblot analysis. The IgE-binding to proteins from both adult and larval ticks was strongly diminished in Swedish and in US serum pools, revealing α -Gal as the main IgE target (Figure 1A,B lanes with α -Gal+) in both populations. Interestingly, proteins at the similar size were identified in *I. ricinus* saliva that bound red meat allergic patients' IgE and were recognized by the anti- α -Gal antibody (Figure 1C). Similar results have been reported for the analysis of saliva from the Japanese tick *H. longicornis*.⁶ In addition, we investigated the IgE-binding capacity with IgE-inhibition ELISA where protein extract from *I. ricinus* was able to inhibit IgE binding to HSA- α -Gal by up to 77% (Figure S2). We also evaluated the allergenic potential of *I. ricinus* using blood from 14 mammalian meat allergic patients and noted that protein extract from adult ticks induced basophil activation in 13 patients (Figure 1D and Figure S3). The allergenic activity towards ticks was higher compared with HSA- α -Gal; however, HSA- α -Gal showed to be more sensitive. This shows that *I. ricinus* protein epitopes also are of importance in basophil activation, which is in line with the IgE-binding capacity results (immunoblots and ELISA). When blood samples from four patients were stimulated with extract from *I. ricinus* larvae (Figure 1E), the allergenic activity was found to be similar to adult *I. ricinus* protein extract in three patients. None of the antigens activated basophils in two nonallergic individuals (Figure S3), indicating that the observed reactions were IgE-dependent. Basophil activation with adult *I. ricinus* protein extract was dose-dependent reaching 72.7% of CD63-positive cells (median, 34.4%; range, 7.5% to 72.7%, at concentration 50 μ g/mL) giving a sensitivity of 93%. Furthermore, a strong correlation between %CD63-positive basophils for adult *I. ricinus* protein extract and HSA- α -Gal (Figure S4) was noted, pointing out the dominant role of the α -Gal epitope in activating red meat allergic patients' basophils.

We used an allergenomics approach with 2D PAGE and 2D immunoblots together with mass spectrometry to identify α -Gal-carrying IgE-binding proteins in adults and larvae ticks (Figure S5 and Figure S6; for details, please see Appendix S1). Analysis of the obtained MS/MS spectra gave high identification scores to 43 protein accession numbers for adult and 37 for larvae from the *Ixodida* order (Table S2), grouped into six protein groups: vitellogenins, SERPIN, actin, α -2-macroglobulin, chitinase-like lectins and transport or

Supplementary Material to:

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Materials and Methods

Animals

In vivo studies in male Wistar rats (approx. 2 months; body weight 350 g $\pm$ 65 g, Charles River Labs, Sulzfeld) were conducted by preclinics GmbH (Potsdam, Germany). Rats were randomly assigned to treatment groups (no allocation parameter) and were allowed free access to tap water and standard diet [R/M-H, extruded (V1536), Ssniff, Soest, Germany]. The animals were kept under 12 h/12 h light–dark cycle conditions. After 19 days of acclimatisation following arrival, animals were anesthetized with 5 vol% isoflurane (IsoFlo 100%; Ecuphar GmbH, Greifswald) and blood was collected from the lateral tail vein to obtain the pre-treatment value. Thereafter, treatment preparation or vehicle solution was administered according to the schedule described under treatment.

The animal study was performed in compliance with the German animal protection law according to the EU Directive 86/609/EEG 1986 and was notified to the Landesamt für Umwelt, Gesundheit und Verbraucherschutz Brandenburg. Housing and handling of the animals were carried out in accordance with EU Directive 2010/63/EU for animal experiments and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (European Treaty Series, ETS, No.123, 1986).

Treatment preparations

Vials with the highest marketed strength of each of two SCIT products marketed in several EU member states were purchased at a local pharmacy. P1 is a preparation of native grass pollen allergens adsorbed to commercial Alhydrogel® adjuvant (100,000 standardized quality units (SQ-E)/mL; Al content: 1.13 mg/mL).¹ P2 is a mixture of allergens from 6 grass pollen species chemically modified with formaldehyde to produce an allergoid, which is then co-precipitated with aluminium hydroxide (10,000 therapeutic units (TU)/mL; Al content: 1 mg/mL).² Plain AH adjuvant suspension (pAH) was prepared from commercial Alhydrogel® 2% (Brenntag Biosector A/S, Frederikssund, Denmark) by dilution with sterile saline to achieve an Al concentration of 1.25 mg per 1 mL. This represents the maximum Al amount allowed in a human dose of allergen products according to the European Pharmacopoeia.³ pAH suspension was freshly prepared within 24h and thoroughly vortexed before administration.

Treatment

Each rat received a single SC injection of 1 mL into the flank of either self-prepared plain AH-adjuvant suspension (group pAH), or one of two SCIT products (groups P1 and P2; see Table 1). A control group receiving SC injection of saline (vehicle group) were run in parallel to monitor the underlying plasma Al steady-state concentration over time (“baseline”) resulting from dietary Al intake. Maximum Al exposure resulting from 1 mL vehicle solution (5 ng) was considered negligible.

Sample collection

Blood samples (approx. 300 μL) were collected from the lateral tail vein at pre-dose, and at day 1, 5, 10, 15, 20, 30, 45, 60, and 80 post-dose using K3-EDTA Multivette 600 collection tubes (Sarstedt, Nümbrecht) connected to a 23G cannula. Blood was stored on ice, centrifuged at 4°C for 10 min at 3220 x g. Plasma was pipetted into 1.5 mL microtubes and stored at – 20°C. In all rats, at time of euthanasia (80 d p.i.) one (right) hemisphere of the brain and the humerus bone of the right front leg were dissected, transferred into 5 mL tubes, weighed, and stored at -70°C. Furthermore, an area of 4x4 cm skin around the injection site was cut, and the subcutis was detached from this skin part and collected as the other tissues. The presence of nodules in the subcutis samples was documented.

Contamination control

To avoid contamination only plastic materials were used (except cannulas and scalpels made of stainless steel and considered to be Al free). Draining tubes (K3-EDTA Multivette 600, Sarstedt, Nümbrecht), which are known to be free of Al, were used for blood sampling. All laboratory ware in contact with the blood sample after drawing (pipette tips, plasma and tissue sample tubes) was immersed for at least 5 min in a 1% (v/v) HNO_3 solution and was allowed to air dry completely. To prevent contamination by air, all steps of decontamination, preparation of samples and reagents were carried out under a clean bench.

Bioanalytical method

Al in tissue samples and plasma was analysed by atomic absorption spectrometric (AAS) procedures. All determinations in the analytical laboratory were conducted in blinded manner.

Tissue samples (0.1 – 2 g) were transferred to ceramic dished plates and the wet weight was determined by an analytical balance. Subsequently, tissue materials were dried at 75°C for 10 h. Dry weight of the samples was determined and dried tissue samples were transferred to volumetric flasks for digestion. After addition of 5 ml HNO_3 (65 %) and 1 ml HCl (30 %) the samples remained at room temperature for 3 hours. The digestion was carried out in a microwave device (Multiwave 3000, Anton Paar, Graz, Austria) at 1400 W for 45 minutes. Ultrapure water was added to the clear acidic residue up to the 15 ml mark. The aliquots were diluted with 0.5% HNO_3 solution by the factor 1:1, 1:10 and 1:100, respectively, according the expected exposure levels. The AAS analysis was performed as described below. Calibration of the tissue digestion solution concentrations was performed by external calibration using blanks and 5 calibrations standards of Al (5, 10, 20, 30, 40, 50 $\mu\text{g/L}$) in 0.5 % HNO_3 .

Plasma samples (100 μL) were diluted by the addition of 100 μL 0.1 % Triton-X solution and 800 μL 0.5 % HNO_3 . Calibration of plasma concentrations was performed by external calibration using bovine plasma as matrix, which was spiked at 5, 10, 20, 40 and 60 $\mu\text{g/L}$. Quantitative determination of the Al concentrations was performed according to the method published by Fleischer and Schaller⁴ using electrothermal atomic absorption spectrometry (AAAnalyst 600, PerkinElmer, Überlingen, Germany). For AAS analysis, 30 μL of the prepared plasma sample, prepared tissue digestion solution, calibration standard, blank and quality control samples, respectively, were injected into the graphite furnace platform. The temperature programme started with the drying phase (ramp time 10 s, 110°C, hold time 25 s; ramp time 40 s, 130°, hold time 25 s), followed by the charging phase (ramp time 15 s, 1350°C, hold time 20 s), atomization phase (ramp time 0 s, 2350°C, hold time 3 s) and

heating phase (ramp time 1 s, 2500°C, hold time 3 s). For the absorption measurement a wavelength of 309.3 nm, a spectral slit width of 0.7 nm, Zeeman background compensation, and peak area analysis was used. The limit of detection was 0.5 µg/L for plasma and 0.2 µg/L for digestion solution analysis, respectively.

In order to avoid extraneous contamination, all instruments and vessels were cleaned by washing with 0.5 % HNO₃ and high precaution was followed in all steps of the procedures. Moreover, reagents blanks were analysed repeatedly in each series. For quality assurance purposes, quality control material (spiked matrix blanks) was analysed in each series and assessed by quality control chart analysis. Analytical accuracy of plasma analysis was assured by the successful participation at GEQUAS proficiency test runs.⁵ Al concentration in bone was determined as µg/g wet weight (ww), in subcutis and brain samples as both µg/g ww and µg/g dry weight (dw).

Data analysis

Individual area under the curve (AUC) of Al in plasma from zero to day 80 (AUC_(0-80d)) was calculated by the linear trapezoidal rule (MS excel).

Total Al amount at injection site on day 80 p.i. was calculated by multiplication of Al concentration measured in subcutis samples with sample wet weight. Subtracting mean Al amount of the vehicle group from individual Al amounts gave an estimate for the individual difference Al amount “remaining” from adjuvant injection. Relating this to the nominal Al dose injected on day 0 gave the percentage of Al dose “remaining” (%). Al dose “released” (%) was calculated as 100 – Al dose “remaining” (%). Absolute “Al amount released” in µg per rat was calculated as % Al dose “released”/100 multiplied by Al dose injected. Negative ratios were not set to zero.

Statistical analysis

If not otherwise indicated, data are presented as means ± standard deviation (SD). Statistical tests were calculated for a two-sided significance level α=0.05, adjusted for multiple comparisons where necessary.

To investigate stability of Al plasma concentration in vehicle group over time, a linear trend curve was fitted to the data from day 0 up to day 80 by means of a linear model for repeated measures (animal) with fixed factor day.

Testing for a significant difference of Al plasma exposure after treatment compared to the vehicle group was done by comparison of total AUC_(0-80d) (Wilcoxon-Mann-Whitney test, two-sided). Percent remaining Al concentration at injection site was tested for a significant difference from 100% by the Wilcoxon signed rank test.

Al concentration in bone or brain samples was compared between groups using a linear model (ANOVA) with fixed factor “treatment” based on logarithmised values. The statistical analysis was performed with SAS®/STAT software, version 9.4, SAS System for Windows, and software R.

Linear regression and correlation (Pearson r) analysis were done by GraphPad Prism® (Version 7.04) software.

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Table 1: Overview of study groups and treatment

Group ID	Animals per group (N)	Treatment	Route of administration	Injection volume	AI dose (mg per animal)	AI dose (mg/kg)
pAH	6	Alhydrogel®-suspension	SC	1 mL	1.25	3.6
P1	6	Adjuvanted allergen product 1 (grass mix), highest marketed strength	SC	1 mL	1.13	3.2
P2	6	Adjuvanted allergen product 2 (grass mix), highest marketed strength	SC	1 mL	1.0	2.9
vehicle	3	saline	SC	1 mL	--	--

Table 2: Al plasma AUC_(0-80d) and Al amounts measured in injection site subcutis, bone and brain on day 80 after injection of AH, P1, P2, or vehicle in rats (mean and standard deviation (SD); coefficient of variance (CV); geometric mean (GM))

Treatment group	Al dose injected (µg)	Al Plasma AUC _(0-80d) (µg/L*d)	injection site subcutis				Bone Al concentration (µg/g ww)	Brain Al concentration (µg/g ww [dw])
			Total Al amount (µg)	Al dose remaining (% injected dose)	Al dose released (% injected dose)	Al amount released (µg per rat)		
pAH (n=6)								
Mean	1250	959	1229.0	98.3	1.7	21.4	0.72	0.20 [‡] [0.75]
SD		115	118.4	9.5	9.5	118.4	0.10	0.28
(CV%)		(12.0)	(9.6)				(13.3)	(137.3)
GM		953	1223.9				0.71	0.08 [0.59]
Stat		<i>p</i> =0.91 [†]		<i>p</i> =0.84 [†]			<i>p</i> <0.001 [§]	<i>p</i> =0.99 [§]
P1 (n=6)								
Mean	1130	992	1003.8	88.8	11.2	126.6	0.64	0.29 [‡] [1.14]
SD		214	149.8	13.3	13.3	149.8	0.12	0.39
(CV%)		(21.5)	(14.9)				(18.5)	(133.8)
GM		976	992.9				0.63	0.18 [0.62]
Stat		<i>p</i> =0.91 [†]		<i>p</i> = 0.03 [†]			<i>p</i> =0.004 [§]	<i>p</i> =0.54 [§]
P2 (n=6)								
Mean	1000	1431	341.3	34.1	65.9	659.1	1.28	0.07 [0.23]
SD		314	222.8	22.3	22.3	222.8	0.26	0.03
(CV%)		(22.0)	(65.3)				(20.5)	(44.7)
GM		1405	148.0				1.26	0.06 [0.21]
Stat		<i>p</i> =0.02 [†]		<i>p</i> = 0.03 [†]			<i>p</i> <0.001 [§]	<i>p</i> =0.96 [§]
Vehicle (n=3)								
Mean	-- [¶]	909	0.40	--	--	--	0.40	0.08 [0.27]
SD		48	0.03				0.11	0.01
(CV%)		(5.3)	(8.3)				(27.8)	(7.2)
GM		908	0.40				0.39	0.08 [0.27]

[†] Wilcoxon test (two-sided) on difference to vehicle group or to 100%

[‡] n=5 only

[§] Evaluation based on linear model for logarithmised values with fixed factor treatment compared to vehicle group.

[¶] < 0.005 µg (see Methods)

Figure 3. Mean (+SD) Al amount (difference to vehicle group mean) found in injection site subcutis of rats (n=6) 80 days after SC injection of pAH, P1, or P2 (light/coloured bars) in comparison to the Al amount injected on day 0 in each group (black bars). Numbers on bars represent the mean percentage of injected dose. * $p < 0.05$ (Wilcoxon signed rank test on difference to 100%)

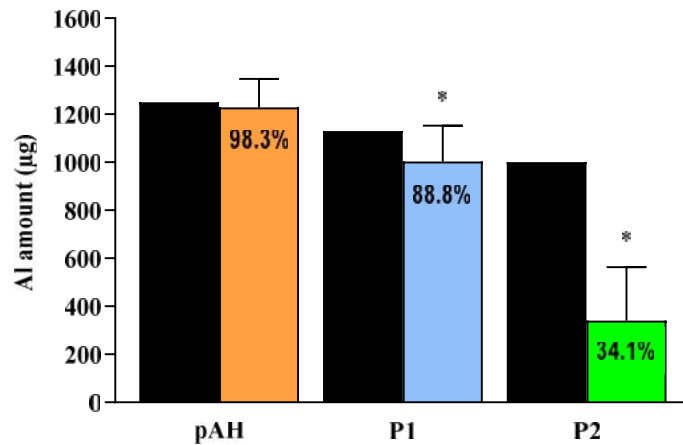


Figure 4. Relationship between Al amount released from injection site and (A) Al plasma AUC_(0-80d) or (B) bone Al concentration on day 80 after injection in individual rats (solid line: linear regression curve; dotted lines: 95% confidence limits)

