The basophil activation test differentiates between patients with alpha-gal syndrome and asymptomatic alpha-gal sensitization

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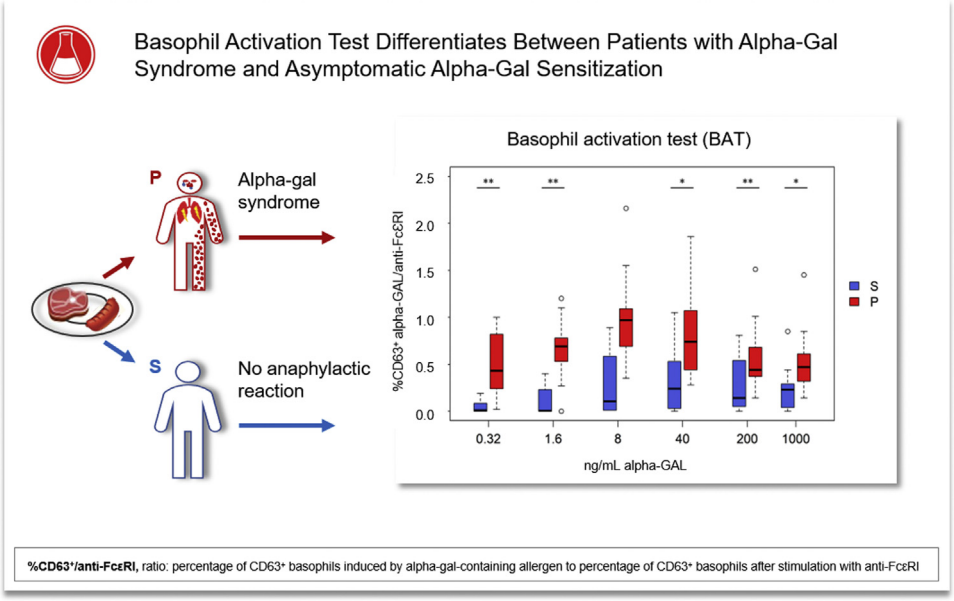
GRAPHICAL ABSTRACT



Background: Galactose-alpha-1,3-galactose (alpha-gal) syndrome is characterized by the presence of serum specific IgE antibodies to alpha-gal and delayed type I allergic reactions to the carbohydrate alpha-gal after consumption of mammalian (red) meat products and drugs of mammalian origin. Diagnostics currently rely on patient history, skin tests,

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determination of serum specific IgE antibodies, and oral food or drug challenges.

Objective: We sought to assess the utility of different basophil parameters (basophil reactivity and sensitivity, the ratio of the percentage of CD63$^+$ basophils induced by the alpha-gal–containing allergen to the percentage of CD63$^+$ basophils after stimulation with anti-FcεRI antibody [%CD63$^+$/anti-FcεRI], and area under the dose-response curve [AUC]) as biomarkers for the clinical outcome of patients with alpha-gal syndrome compared with subjects with asymptomatic alpha-gal sensitization.

Methods: In addition to routine diagnostics, a basophil activation test (Flow CAST) with different concentrations of alpha-gal–containing allergens (eg, commercially available alpha-gal–carrying proteins and pork kidney extracts) was performed in 21 patients with alpha-gal syndrome, 12 alpha-gal–sensitized subjects, and 18 control subjects.

Results: Alpha-gal–containing allergens induced strong basophil activation in a dose-dependent manner in patients. Basophil reactivity at distinct allergen concentrations, the %CD63$^+$/anti-FcεRI ratio across most allergen concentrations, the AUC of dose-response curves, and basophil allergen threshold sensitivity (CD-sens) with pork kidney extract were significantly higher in patients with alpha-gal syndrome compared with those in sensitized subjects. All parameters were negative in control subjects.

Conclusion: The basophil activation test should be considered as an additional diagnostic test before performing time-consuming and potentially risky oral provocation tests. The %CD63$^+$/anti-FcεRI ratio for all allergens and AUCs for pork kidney were the best parameters for distinguishing patients with alpha-gal syndrome from subjects with asymptomatic alpha-gal sensitization. (J Allergy Clin Immunol 2019;143:182-9.)

Key words: *Basophil activation test, alpha-gal syndrome, galactose-alpha-1,3-galactose, red meat allergy, CD63, CCR3, basophil allergen threshold sensitivity (CD-sens), asymptomatic alpha-gal sensitization*

Abbreviations used

AHSG:	Human alpha 2–Heremans-Schmid glycoprotein
alpha-gal:	Galactose-alpha-1,3-galactose
AUC:	Area under the curve
BAT:	Basophil activation test
%CD63$^+$/anti-FcεRI:	Ratio of the percentage of CD63$^+$ basophils induced by the alpha-gal–containing allergen to the percentage of CD63$^+$ basophils after stimulation with anti-FcεRI antibody
CD-sens:	Basophil allergen threshold sensitivity
EC50:	Half-maximum effective concentration or effective dose at 50% of the maximum dose response
P patients:	Patients with alpha-gal syndrome, diagnosed by positive oral food challenges or presenting a very convincing medical history of systemic type I reactions of severity grades I to III after exposure to alpha-gal–containing substances
ROC:	Receiver operating characteristic
sIgE:	Serum specific IgE
SPT:	Skin prick test
SPPT:	Skin prick-to-prick test
S subjects:	Sensitized subjects with sIgE to alpha-gal values of 0.10 kU/L or greater without an explicit medical history of allergic type I reactions to mammalian red meat products

Anaphylactic reactions after consuming red meat have previously been described in rare individual cases.[1-4] Major allergens described for any kind of meat allergy were serum albumins, immunoglobulins, and muscle proteins.[5] It was Commins et al,[6] in 2009, who reported a novel form of delayed anaphylaxis to red meat related to serum specific IgE (sIgE) antibodies to the oligosaccharide galactose-alpha-1,3-galactose (alpha-gal).

The prevalence of this so-called alpha-gal syndrome[7,8] varies in different countries. This new type of food allergy was first detected in patients living in southeastern regions of the United States and Australia, but soon thereafter, it was also recognized in Europe, Asia, Africa, and Central America.[8-11] The disease is clearly associated with tick bites.[12-14] Therefore German forest service employees and hunters comprise a population with a considerable risk (odds ratio, 2.48) of red meat allergy compared with the residential population, with 8.6% of sensitized subjects having mammalian meat–induced food allergy in a recent study.[15]

The diagnosis of this allergic disease is based on a history with a typical delayed reaction (3-6 hours) after consumption of red meat. However, sometimes an immediate reaction (<1 hour) is found to depend on the allergen source (innards, such as pork kidney); cofactors, such as exercise, alcohol, or nonsteroidal anti-inflammatory drugs; and comorbidities, such as mastocytosis.[8,16,17] Skin tests with alpha-gal–containing substances (eg, fresh meat, kidney preparations, and cetuximab), determination of alpha-gal sIgE levels, and oral provocation test results with muscle meat or pork kidney combined with cofactors confirm the diagnosis.[8] In addition to these patients with alpha-gal syndrome, subjects have been identified in screening tests (eg, for idiopathic anaphylaxis) to have type I sensitization to alpha-gal but to clearly tolerate red meat.[14] For example, German forest service employees and hunters showed a prevalence of alpha-gal sIgE positivity (≥0.10 kU_A/L) of 35%, but only 4.8% had a red meat allergy.[15]

It is uncertain whether asymptomatic alpha-gal–sensitized subjects have a risk of alpha-gal syndrome or are just sensitized without a clinically relevant allergy. Food challenges can be used to distinguish between allergy and sensitization, but these tests are time-consuming and can cause severe anaphylactic reactions. Also, the delayed appearance of symptoms requires a long patient observation period. Anaphylaxis was observed after challenge with very low amounts (eg, 3 g) of pork kidney[16] in patients with alpha-gal syndrome. Furthermore, subjects without a typical history would not be motivated to submit themselves to oral provocation tests.

During the last decade, the basophil activation test (BAT) has been shown to be a useful *in vitro* diagnostic method for evaluation of food allergy. Basophils are identified with unique basophil markers, and their activation is measured fluorometrically by using activation markers (eg, CD63). The number of basophils that respond to a given dose of the stimulus is defined as basophil reactivity. Also, other parameters (eg, basophil allergen threshold sensitivity [CD-sens], half-maximum effective concentration or effective dose at 50% of maximum dose response [EC50], and area under the curve [AUC]) can be calculated from a given dose-response curve. Basophil sensitivity is a

TABLE I. Characteristics of patients with alpha-gal syndrome (P patients) and subjects sensitized to alpha-gal (S subjects)

Group	No.	Age (y)		Male subjects		Trigger*		Clinical reaction severity grades I, II, and III†		sIgE alpha-gal (≥0.1 kU/L)		Positive prick-to-prick skin test response to alpha-gal–carrying substances‡		Oral provocation test					
														Not done		Positive		Negative	
				No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
P patient	21	46.5	26-56	13	62	21	100	21	100	21	100	19	90	15	71	5	24	1§	5
S subjects	12	54.2	30-60	9	75	0	0	0	0	12	100	7	58	9	75	0	0	3	25

*Pork kidney, pork, or beef.
†Anaphylactic reaction caused by pork kidney, pork, or beef.
‡Pork kidney and meat (raw and cooked) or gelatin; beef kidney and meat (raw and cooked), gelatin, or cow's milk; cetuximab (500 μg/mL); or Gelafundin (4%).
§Oral provocation not fully completed.

TABLE II. Characteristics of the control groups not sensitized to alpha-gal (with and without atopy)

Group	No.	Age (y)		Male subjects		Atopic eczema		Asthma		Allergic rhinitis		sIgE (>0.35 kU/L) to atopic screening*		Positive skin test response to atopic screening†	
				No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent	No.	Percent
CA	10	37.4	26-56	2	20	3	30	4	40	7	70	4	40	8	80
C0	8	38.8	30-60	3	38	0	0	0	0	0	0	0	0	0	0

CA, Control group with atopy; *C0*, control group without atopy.
*Grass, birch, Bet v 1, *D pteronyssinus*, and cat.
†Grass, birch, *D pteronyssinus*, and cat.

function of reactivity and the compound affinity of cell-bound sIgE for allergens and free competing immunoglobulins. It has been shown that these latter parameters can be sensitive biomarkers reflecting the clinical severity of anaphylactic reactions, the clinical threshold for eliciting symptoms, and the therapeutic effects of, for example, immunotherapy.[18] Especially in patients with food allergy, an additional value of the BAT was shown: in patients with cow's milk allergy, the test was useful in assessing the natural resolution of this allergy in children.[19] In a study of egg oral immunotherapy, there was a correlation between basophil suppression and clinical desensitization.[20] Basophil sensitivity was able to discriminate between subjects sensitized to hazelnut and allergic subjects.[21] The most convincing results were shown with peanut allergy. It could be shown that basophil reactivity was associated with severity and basophil sensitivity with the threshold of allergic reactions to peanut.[22]

It was the aim of this study to assess whether distinct parameters of the BAT could contribute to a differentiation between a clinically relevant alpha-gal syndrome (patients with alpha-gal syndrome diagnosed based on positive oral food challenge results or presenting a very convincing medical history of systemic type I reactions of severity grades I to III after exposure to alpha-gal–containing substances [P patients]) and a sensitization to alpha-gal without clinical relevance (sensitized subjects with sIgE to alpha-gal values of ≥0.10 kU/L without an explicit medical history of allergic type I reactions to mammalian red meat products [S subjects]). Use of such parameters could reduce or avoid oral provocation tests. Furthermore, recommendations for a diet without red meat and the advice to avoid alpha-gal–containing drugs could be given based on the test results.

METHODS

Study population

A total of 51 subjects were recruited from participating medical centers. Thirty-three of them had alpha-gal sensitization, which was defined as values of specific alpha-gal IgE of 0.10 kU/L or greater, according to a prior study.[15]

Twenty-one patients had alpha-gal syndrome (P patients) and were given a diagnosis based on positive oral food challenge results or presented with a very convincing medical history of systemic type I reactions of severity grade I to III (according to the Ring and Messmer classification[23]) after exposure to alpha-gal–containing substances (predominantly red meat products). Twelve sensitized subjects with sIgE to alpha-gal values of 0.10 kU/L or greater presented themselves for allergological clarification after anaphylactic reactions but had no explicit medical history of allergic type I reactions to mammalian red meat products (S subjects). For details, see Table I and Table E1 in this article's Online Repository at www.jacionline.org. Twenty of 21 P patients and 10 of 12 S subjects remembered tick bites (species unknown).

Because of the possibility of subclinical basophil activation after consumption of dairy products in alpha-gal–sensitized subjects, participants were encouraged to follow a 48-hour abstinence of meat and dairy products before blood sampling.

The control population without specific alpha-gal IgE antibodies (n = 18) consisted of an atopic and a nonatopic group. For details, see Table II.

The study protocol was approved by the ethics committees of all medical centers (Technische Universität München, Eberhard Karls Universität Tübingen and the National Committee for Medical Research Ethics Luxembourg), and written informed consent was obtained from all participants.

Skin tests, sIgE measurements, and total IgE and tryptase levels

Skin prick tests (SPTs) were performed with commercially available pork, beef, and milk extracts, as well as grass, birch, *Dermatophagoides pteronyssinus*, and cat allergens (Allergopharma GmbH & Co. KG, Reinbek, Germany,

and ALK-Abelló A/S, Hørsholm, Denmark), for atopic screening. Skin prick-to-prick tests (SPPTs) were carried out with pork and beef kidney, raw and cooked pork and beef, pork gelatin, bovine gelatin, cow's milk, the drugs cetuximab (500 μg/mL; Erbitux, Merck KGaD, Darmstadt, Germany), and the succinylated gelatin volume expander Gelafundin 4% (B. Braun; Melsungen AG, Melsungen, Germany). Intracutaneous tests with Gelafundin 4% were performed when SPTs produced negative responses. Serum sIgE levels against alpha-gal (o215), pork (f26), beef (f27), cow's milk (f2), grass (g6), birch (t3), Bet v1 (t215), *D pteronyssinus* (D1), cat (e1), and rFel d 2 cat serum albumin (e220), total IgE and tryptase levels were measured with an immunoenzymatic assay (ImmunoCAP250 and Phadia; Thermo Fisher Scientific, Waltham, Mass).

Reagents for BATs

Pork kidney extract was prepared with pork kidney purchased at a local retailer and cut into small pieces. Lysis buffer (50 mmol/L Tris HCl, pH 8) containing protease inhibitors (Protease Inhibitor Cocktail Tablets; Roche, Mannheim, Germany) was added, and tissue was disrupted with beads (Tissue Lyser II; Qiagen, Hilden, Germany). Particles were eliminated by means of centrifugation for 10 minutes at 20,000*g*. The final protein concentration was determined by using the Bradford Protein Assay (Bio-Rad, Nazareth, Belgium). Gal-α-1,3-Gal-β-1,4-GlcNAc-HSA (alpha-Gal-HSA; NGP 2334), a linear trisaccharide, was obtained from Dextra Laboratories (Reading, England). Alpha-GAL (BAG2-GAL), pork (BAG2-F26), and beef (BAG2-F27) were provided by BÜHLMANN Laboratories AG (Schönenbuch, Switzerland). Human alpha 2–Heremans-Schmid glycoprotein (AHSG; fetuin-A in animals) was recombinantly produced and engineered to carry alpha-gal. Details of the production and characterization of this alpha-Gal–AHSG can be found in the Methods section in this article's Online Repository at www.jacionline.org. Concentrations of all alpha-gal–containing reagents used are shown in Table III.

BATs

The Flow CAST (BÜHLMANN Laboratories AG) was used for quantitative measurement of *in vitro* basophil activation, as previously described.[24] Venous blood was collected in 10-mL EDTA tubes from each subject. For each subject and allergen, polystyrene tubes were prepared with 50 μL of allergen at the defined concentration (Table III) and diluted in 100 μL of stimulation buffer (containing heparin, Ca^{2+}, and IL-3 [2 ng/mL]). An anti-FcεRI mAb and anti–N-formyl-methionyl-leucyl-phenylalanine were used as positive controls. Background values were assessed with 50 μL of stimulation buffer.

We added and gently homogenized 50 μL of each subject's whole blood (with EDTA), added 20 μL of staining reagent (anti-CD63–fluorescein isothiocyanate and anti-CCR3–phycoerythrin mAbs), and mixed and incubated the solution at 37°C for 25 minutes in an incubator. Two milliliters of lysing buffer stopped the stimulation within 5 minutes of incubation in the dark at room temperature, followed by centrifugation of the solution for 5 minutes at 460*g*. The supernatant was decanted, and stained cells were washed with 300 μL of washing buffer. Cells were resuspended by means of gentle vortexing and analyzed by using FACScan or LSR Fortessa flow cytometers (Becton Dickinson Immunocytometry System; Becton Dickinson, Heidelberg, Germany) with BD CellQuest or BD FACSDiva software.

Basophils were gated as low side scatter $CCR3^{+}$/side $scatter^{low}$. Anti-CCR3 was used as a selection marker to separate basophils from other leukocytes. Upregulation of the basophil activation marker CD63 was calculated based on the percentage of $CD63^{+}$ cells compared with the total number of identified basophils. In each assay a minimum of 300 events (ie, basophils) were recorded. A cutoff of 15% $CD63^{+}$ cells was used as recommended for food allergens by the supplier and according to a previous study.[25]

Determination of different BAT parameters

The following parameters were determined: basophil reactivity and maximum percentage $CD63^{+}$ (highest value of activated basophils by 1 allergen); the ratio of the percentage of $CD63^{+}$ basophils induced by the alpha-gal–containing allergen to the percentage of $CD63^{+}$ basophils after stimulation with anti-FcεRI mAb (%CD63 $allergen^{+}$/anti-FcεRI); CD-sens, the inverse value of the concentration at half-maximum stimulation (ie, EC50) multiplied by 100; and the AUC.

TABLE III. Concentrations of the tested allergens in BATs

Allergen	Concentration after reconstitution	Concentration in stimulation
Pork kidney extract (μg/mL)	100	22.7
	10	2.27
	1	0.227
	0.1	0.0227
	0.01	0.00227
	0.001	0.00022
Alpha-Gal–HSA (ng/mL)*	10,000	2,272
	1,000	227
	100	22.7
	10	2.27
	1	0.22
	0.1	0.022
Alpha-GAL (ng/mL)†	4,400	1,000
	880	200
	176	40
	35.2	8
	7.04	1.6
	1.41	0.32
AHSG (ng/mL)	1,000	227.3
Pork (ng/mL)†	100	22.7
	25	4.5
	5	0.9
Beef (ng/mL)†	100	22.7
	25	4.5
	5	0.9

*Purchased from Dextra Laboratories, Reading, United Kingdom.
†Provided by BÜHLMANN Laboratories AG, Schönenbuch, Switzerland.

Statistical analysis

Statistical analyses were carried out with SPSS Statistics software (IBM, New York, NY) and R software (R Development Core Team, Vienna, Austria) with the Epi package for epidemiologic analysis. Continuous variables are presented as medians and ranges. Distributions of relevant groups were compared with Mann-Whitney *U* tests. The Fisher exact test was applied to compare categorical SPT results. Receiver operating characteristic (ROC) analyses were performed to estimate the discriminatory ability of the investigated parameters. ROC AUCs are presented. Box plots were created in R software by using the Epi package to illustrate distribution of relevant variables within groups. All statistical tests were performed 2-sided, and a significance level of 5% was used.

RESULTS

Study population

Thirty-three sensitized patients were included in the study, and 21 of them had alpha-gal syndrome. Of these 33 subjects, 7 (6 with alpha-gal syndrome) were excluded because they were nonresponders to anti-FcεRI antibody (n = 4) or revealed high background values (>10% CD63 activation) in the BAT (n = 3 P patients). Accordingly, 26 subjects (15 P patients and 11 S subjects; 18 male and 8 female subjects; age range, 13-76 years; median age, 52.5 years) were analyzed further.

The healthy control population (n = 18) consisted of 2 groups of nonsensitized subjects. Of the 10 nonsensitized subjects with atopy,

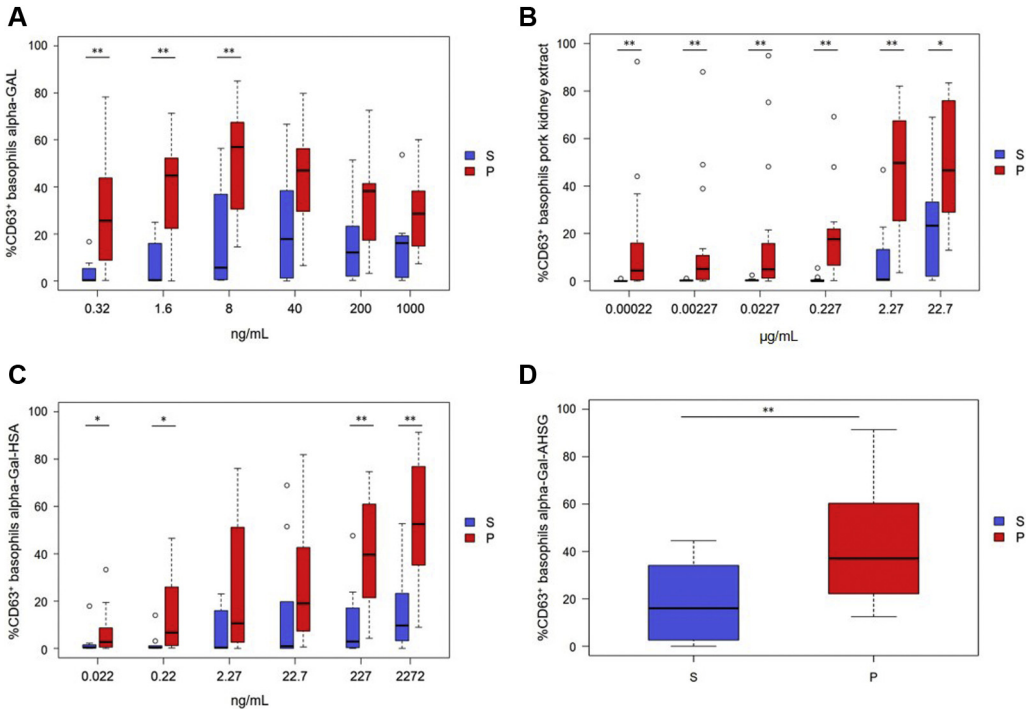

FIG 1. Dose-dependent basophil activation in patients with alpha-gal syndrome (P patients) and alpha-gal sensitized subjects (S subjects) by using alpha-GAL (BÜHLMANN Laboratories; **A**), pork kidney extract **(B)**, alpha-Gal–HSA (Dextra Laboratories; **C**), or alpha-Gal–AHSG **(D)**. $*P < .05$ and $**P < .01$ for the comparison between groups using the Mann-Whitney *U* test. ○Individual outliers of less than the first quartile and greater than the third quartile.

1 was excluded as a nonresponder, and 9 participants remained in the study population. All 8 nonsensitized nonatopic subjects were included in the analysis. For details, see Tables I and II.

Skin tests, sIgE measurements, and total IgE and tryptase levels

There were positive SPPT responses for at least 1 allergen in most P patients (19/21; 1 of the negative patients showed a positive intracutaneous test result), revealing a sensitivity of 90%. In the group of S subjects, 7 of 12 subjects had positive SPPT responses. SPPT responses with raw pork kidney were positive in P patients significantly more often (94%) compared with those in S subjects (50%, $P = .04$).

P patients had significantly greater levels of sIgE to alpha-gal ($P = .019$), pork ($P = .013$), beef ($P = .005$), and cow's milk ($P = .018$) and also greater tryptase levels ($P = .008$).

Total IgE levels were not significantly different between the groups. For details, see Tables I and II, Tables E1 and E2, and Fig E1 in this article's Online Repository at www.jacionline.org.

Basophil activation parameters

Basophil reactivity and maximum %CD63$^+$. The pork kidney extract, AHSG, or commercially available alpha-gal–carrying proteins induced basophil activation (>15%) at a minimum of 1 concentration in all 15 P patients (sensitivity of 100%). Basophil activation was dose dependent up to a maximum activation of 94.9% CD63$^+$ (median, 69.0%; range, 22.8% to 94.9%) with pork kidney extract. P patients had significantly higher basophil activation compared with S subjects at distinct allergen concentrations (Fig 1). In 4 S subjects no basophil activation was measured.

Commercially available pork and beef allergen solutions did not induce basophil activation over the cutoff of 15% in any of the 18 analyzed sensitized subjects (P patients and S subjects).

Background values were significantly greater in P patients compared with those in S subjects (median of 2.8% [range, 0.0%-7.3%] vs 0.3% [range, 0.0%-1.2%], $P = .001$). A weak association between tryptase and baseline CD63 values was observed (Spearman rank correlation coefficient $[r] = 0.277$, $P = .125$, $n = 26$).

No activation was detected (specificity 100%) in basophils of the control population.

Comparison of BAT and skin test results. All P patients with positive BAT results also had positive skin test responses. There was no explicit correlation between BAT results and SPPT responses in the group of S subjects. Corresponding results were presented in 9 subjects (positive BAT and SPPT results, 5/11; negative BAT and SPPT results, 4/11), and incongruent results were found in 2 of 11 subjects (1 negative BAT result and positive SPPT response and 1 positive BAT result and negative SPPT response).

%CD63$^+$/anti-FcεRI. The %CD63$^+$/anti-FcεRI ratio was statistically significantly higher at most concentrations in P patients compared with S subjects across all tested allergens (Fig 2).

EC50 and CD-sens values. EC50 and CD-sens values were calculated from the subjects' dose-response curves for all allergens that induced high basophil activation and were used at 6 different concentrations (commercially available

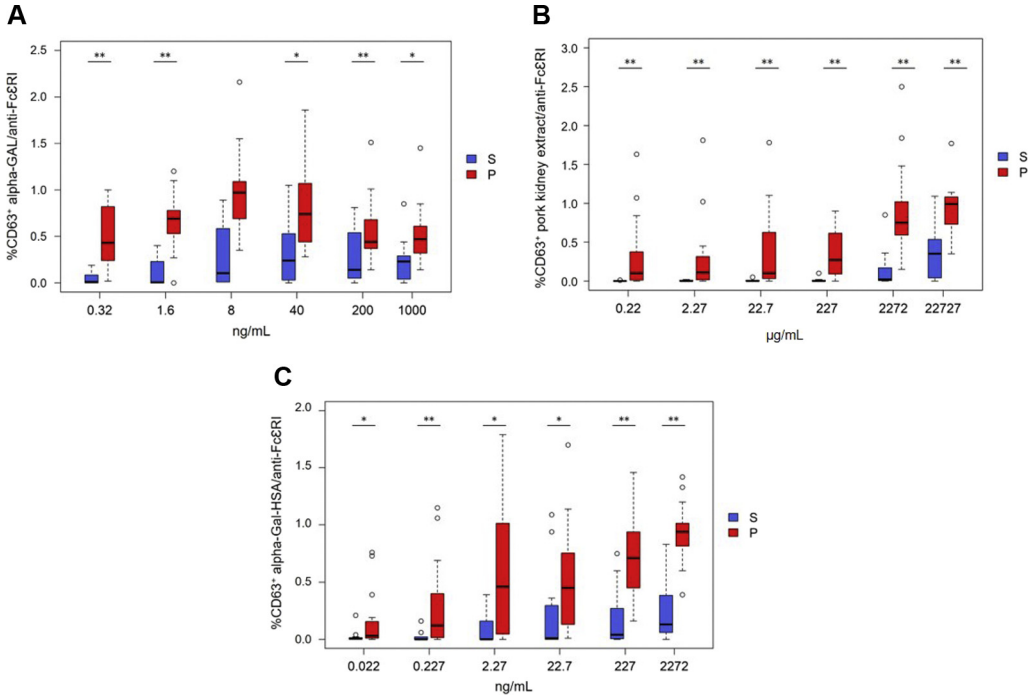

FIG 2. %CD63$^+$ using alpha-GAL (BÜHLMANN Laboratories)/anti-FcεRI **(A)**, %CD63$^+$ using pork kidney extract/anti-FcεRI **(B)**, and %CD63$^+$ using alpha-Gal–HSA (Dextra Laboratories)/anti-FcεRI **(C)** in patients with alpha-gal syndrome (P patients) and alpha-gal–sensitized subjects (S subjects). $*P < .05$ and $**P < .01$ for the comparison between groups using the Mann-Whitney *U* test. ○Individual outliers of less than the first quartile and greater than the third quartile.

alpha-gal–carrying proteins and pork kidney extract). Atypical dose-response curves of the allergens were obtained in some subjects; as a result, no EC50 values could be determined, and subsequently, no CD-sens values were calculated (4/45 curves in P patients and 10/33 curves in S subjects).

The pork kidney extract produced a significantly higher CD-sens value (median of 175.4 [range, 14.5-10,000.0] vs 18.5 [range, 11.1-144.9], $P = .001$) and correspondingly lower EC50 values (median of 0.6 [range, 0.0-6.9] vs 5.7 [range, 0.7-9.0], $P = .001$) in this group (Fig 3). The commercially available alpha-gal–carrying proteins showed a trend toward greater CD-sens values and correspondingly lower EC50 values in P patients compared with S subjects, but statistical significance was not attained except for EC50 with alpha-GAL ($P = .05$), see Fig E2 in this article's Online Repository at www.jacionline.org.

AUC of the dose-response curve. AUCs of the dose-response curves of the commercially available alpha-gal–carrying proteins and pork kidney extract were significantly greater in P patients compared with asymptomatic S subjects. The corresponding values for AUC of alpha-GAL (BÜHLMANN Laboratories) were a median of 141.1 (range, 29.2-236.8) versus a median of 38.8 (range, 0.8-160.9; $P = .006$), those for AUC of alpha-Gal–HSA (Dextra Laboratories) were a median of 135.9 (range, 27.1-296.7) versus a median of 14.1 (range, 0.7-160.7; $P = .001$), and those for AUCs for pork kidney extract were a median of 118.6 (range, 17.8-321.3) versus a median of 12.0 (range, 1.0-79.9; $P = .00015$). Table IV shows the selected threshold values of the abovementioned BAT parameters exceeded by P patients and not reached by S subjects.

AUCs of ROC curves. AUCs of ROC curves determine how well the tests discriminate between the groups. AUC values were greatest for %CD63$^+$ alpha-GAL/anti-FcεRI ratio at 0.32 ng/mL and for %CD63$^+$ alpha-Gal–HSA/anti-FcεRI ratio at 2.272 μg/mL (both 0.952), followed by the AUC for CD63$^+$ activation at 0.227 μg/mL pork kidney extract (0.945) and for %CD63/anti-FcεRI pork kidney extract at 0.227 μg/mL (0.924; data not shown).

AUC analysis of specific IgE antibody determination did not yield particularly good results: the sIgE value for alpha-gal was 0.780, the sIgE value for beef (f27) was 0.832, and the sIgE value for pork (f26) was 0.789.

DISCUSSION

In this study we could confirm the utility of different basophil parameters as biomarkers of the clinical outcome of patients with alpha-gal syndrome (P patients) compared with asymptomatic subjects with alpha-gal sensitization (S subjects). This is an important issue because (1) it is uncertain whether alpha-gal sensitization has clinical relevance, (2) severe reactions during oral provocation tests with small amounts (eg, 3 g) of pork kidney were seen, and (3) onset of the reaction could be delayed by several hours.[8,16]

Oral provocation studies in patients with alpha-gal syndrome revealed *ex vivo* basophil activation 3 to 7 hours after provocation with mammalian meat occurring within the same time frame as clinical symptoms appeared, thus supporting the *in vivo* role of basophils in this type of food allergy or at least their concomitant activation.[25]

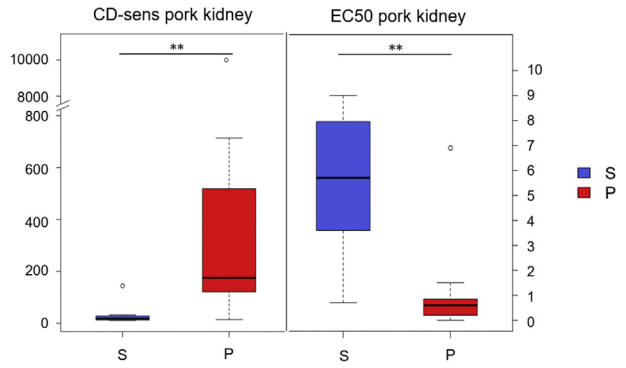

FIG 3. CD-sens and EC50 values using pork kidney extract in patients with alpha-gal syndrome (P patients) and alpha-gal–sensitized subjects (S subjects). $^{**}P < .01$ for the comparison between groups using the Mann-Whitney *U* test. ○Individual outliers of less than the first quartile and greater than the third quartile.

Previous *in vitro* studies have already shown that extracts of pork kidney, an organ with a high amount of alpha-gal–carrying epitopes, are capable of inducing basophil activation.[26,27] We used these preparations and other commercially available and recombinantly produced alpha-gal proteins. With all preparations, patients with alpha-gal syndrome had positive results, whereas atopic and nonatopic control subjects revealed no basophil activation, resulting in excellent sensitivity and specificity of 100% if nonresponders were excluded.

As a positive control, the highly specific mAb binding to anti-FcεRI has been used for several years as a stimulus in BATs, mimicking bridging of the receptor by an allergen. Rubio et al[19] analyzed the ratio between the percentage of $CD63^+$ basophils after incubation with food allergen (here cow's milk protein) and the percentage obtained with the positive control (anti-FcεRI). He was first to show in patients with food allergy that a statistical correlation exists between this ratio, oral challenge outcome (absence/presence of clinical reaction), reaction severity, and eliciting dose of milk in positive challenge results. In patients with peanut allergy, this parameter was associated with the severity of the reaction during standardized oral provocation tests.[22] We could only calculate the difference between S subjects without clinical symptoms because an oral provocation challenge was performed in only 3 of the 12 sensitized subjects and 6 of the 21 P patients. Furthermore, a suggested standardized provocation protocol[8] was not strictly performed in all centers because of different established methods for oral provocation tests in each allergy unit. Nevertheless, the $\%CD63^+$/anti-FcεRI ratio turned out to be one of the best ways to differentiate between the 2 alpha-gal–sensitized groups, with significant differences for almost all tested allergens.

CD-sens was first introduced by Nopp et al.[28] They showed that it is a useful approach to evaluate the efficacy of omalizumab treatment. This parameter (EC50 or CD-sens) was shown to be of value to discriminate between patients with bee and wasp venom allergy with double sensitization, to follow up on specific immunotherapy in patients with insect venom and grass pollen allergy, and to show clinical relevance in patients with food allergy.[22,24,29-32] In our study the calculation of CD-sens with pork kidney extract showed significant differences between P patients and S subjects. The missing significant differences found with the commercially available alpha-gal proteins might be due to difficulties calculating the accurate EC50 in atypical dose-response curves (eg, in cases of high basophil activation already at the lowest allergen concentration). This was also a problem described in a recent study about the data-driven programmatic approach to analysis of BAT results.[33] In those cases the calculation of the area under the dose-response curve can be helpful because it attempts to combine basophil reactivity and sensitivity into 1 parameter and includes partial anergy induced at high allergen concentrations.[34] In our study the area under the dose-response curve, especially with pork kidney extract and also with commercially available alpha-gal proteins, showed significant differences between the 2 groups.

TABLE IV. Selected threshold values of different BAT parameters exceeded by patients with alpha-gal syndrome (P patients) and not reached by alpha-gal–sensitized subjects (S subjects)

Parameter	Threshold value	P patients (> threshold value)	S subjects (> threshold value)
Basophil reactivity ($\%CD63^+$)			
Pork kidney extract (2.27 μg/mL)	46.8	57%	0%
Alpha-GAL (8 ng/mL)	56.4	58%	0%
Alpha-Gal–HSA (2272 ng/mL)	52.7	54%	0%
$\%CD63^+$ allergen/anti-FcεRI			
Pork kidney extract (0.227 μg/mL)	0.100	85%	0%
Alpha-GAL (1.6 ng/mL)	0.395	92%	0%
Alpha-GAL (0.32 ng/mL)	0.194	85%	0%
Alpha-Gal–HSA (2272 ng/mL)	0.833	67%	0%
CD-sens			
Pork kidney extract	144.9	67%	0%
AUC of the dose-response curve			
Pork kidney extract	79.9	73%	0%
Alpha-GAL	160.9	38%	0%
Alpha-Gal-HSA	160.7	47%	0%

Calculation of ROC AUCs provided additional information about the best parameters to use to discriminate between the 2 groups. In our study the best values were obtained by using the $\%CD63^+$ alpha-GAL/anti-FcεRI ratio at the lowest allergen concentration and the $\%CD63^+$ alpha-Gal–HSA/anti-FcεRI ratio at the highest concentration, followed by the AUC with pork kidney extract and $\%CD63^+$ pork kidney extract at 0.227 μg/L. From a practical point of view, commercially available alpha-gal proteins might be easier to use in BATs than pork kidney extract, which requires preparation in advance. Useful threshold values of the different parameters that are only exceeded by P patients can be found in Table IV.

Because basophil background activation and tryptase levels were significantly greater in P patients compared with S subjects, subclinical activation of basophils and mast cells can be assumed.

According to the results of our study, low sIgE values to alpha-gal, pork, or beef together with negative skin test results to raw pork kidney can indicate clinically irrelevant alpha-gal sensitization in the case of a negative history, but values for the ROC

AUC curves for these serologic parameters were lower than with the abovementioned BAT parameters. Therefore, additional BAT parameter thresholds, which point out decreased basophil reactivity and sensitivity, support the clinician in the decision of performing or not performing an oral provocation test. Thresholds of BAT parameters showing increased basophil reactivity and sensitivity could help the clinician choose adequate doses for oral provocation tests in patients with alpha-gal syndrome and advise them to avoid foods and drugs containing very small amounts of alpha-gal.

We thank Franziska Martin for her excellent technical support and Dr Bernhard Haller and Lynne Stecher for their help with statistical analysis. We also thank everyone who participated in the study. We thank BÜHLMANN Laboratories AG, Schönenbuch, Switzerland, for their support with reagents and in particular Michael Schneider for his technical and methodological support with BAT analyses, as well as calculation of CD-sens and AUC values.

Clinical implications: Distinct parameters of the BAT allowed a good differentiation between patients with alpha-gal syndrome and asymptomatic alpha-gal sensitization and should be determined before performing oral provocation tests.

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Methods

Recombinant production of alpha-Gal-AHSG

The coding region of *Mus musculus* alpha-1,3-galactosyltransferase (Ggta1) was amplified from esophagus cDNA by PCR (AccuPrime Pfx DNA Polymerase, Thermo Fisher Scientific, Schwerte, Germany) using primers producing restriction enzyme recognition sites for cloning (forward primer: 5'-GAT CAA GCT TAT GAT CAC TAT GCT TCA AGA TCT C-3'; reverse primer: 5'-GAT CCC GCG GAC ATT ATT TCT AAC CAA ATT ATA CTC-3'). The PCR product was cloned via *Hind*III and *Sac*II into the digested pcDNA3.1/Neo vector. HEK293 cells (cultured in DMEM high glucose, GlutaMAX supplemented with 10% FCS and 50 µg/mL Penicillin/Streptomycin at 37 °C with 5% CO_2) were transfected with the Ggta1-containing vector using Lipofectamin 3000 (Thermo Fisher Scientific) as recommended by the manufacturer. Cells were selected for stable integration of the vector (HEK293-Ggta1) by the addition of G418 (500 µg/mL) to the cell culture medium.

Human alpha(2)-Heremans-Schmid glycoprotein (AHSG) (fetuin-A in animals) was amplified from PBMC cDNA by PCR using primers producing restriction enzyme recognition sites for cloning and an N-terminal His-tag for purification (forward primer: 5'-GAT CAT TTA AAT GTG TCC AGT GTC ACC ATC ATC ACC ATC ACC ATC ATC ACC ATG CCC CAC ATG GCC CAG GG-3', reverse primer: 5'-GAT CTC TAG ATC ACG TAG AAT CGA GAC CG-3'). PCR products were cloned via *Swa*I and *Xba*I into the digested pcDNA3.1/Zeo vector containing an N-terminal human IgG heavy chain signal peptide for secretion and a C-terminal V5-epitope tag for detection. HEK293 and HEK293-Ggta1 cells were transfected with the AHSG-containing vector as described above and selected for stable integration by adding Zeocin (100 µg/mL) to the culture medium.

After expansion of HEK293-AHSG and HEK293-Ggta1-AHSG cells, secreted AHSG and alpha-gal-AHSG were purified from culture supernatants by affinity chromatography. Culture supernatants were loaded on a nickel-chelating affinity matrix (HisTrap excel, GE Healthcare Life Sciences, Freiburg, Germany), the column was washed with phosphate-buffered saline (PBS, pH 8), nonspecifically bound proteins were removed by washing with PBS containing 60 mM imidazole and recombinant proteins were eluted with PBS containing 150 mM imidazole. SDS-PAGE and Western blotting confirmed the purity of the proteins and the presence of alpha-gal epitopes on alpha-Gal-AHSG (Supplemental Fig. 1).

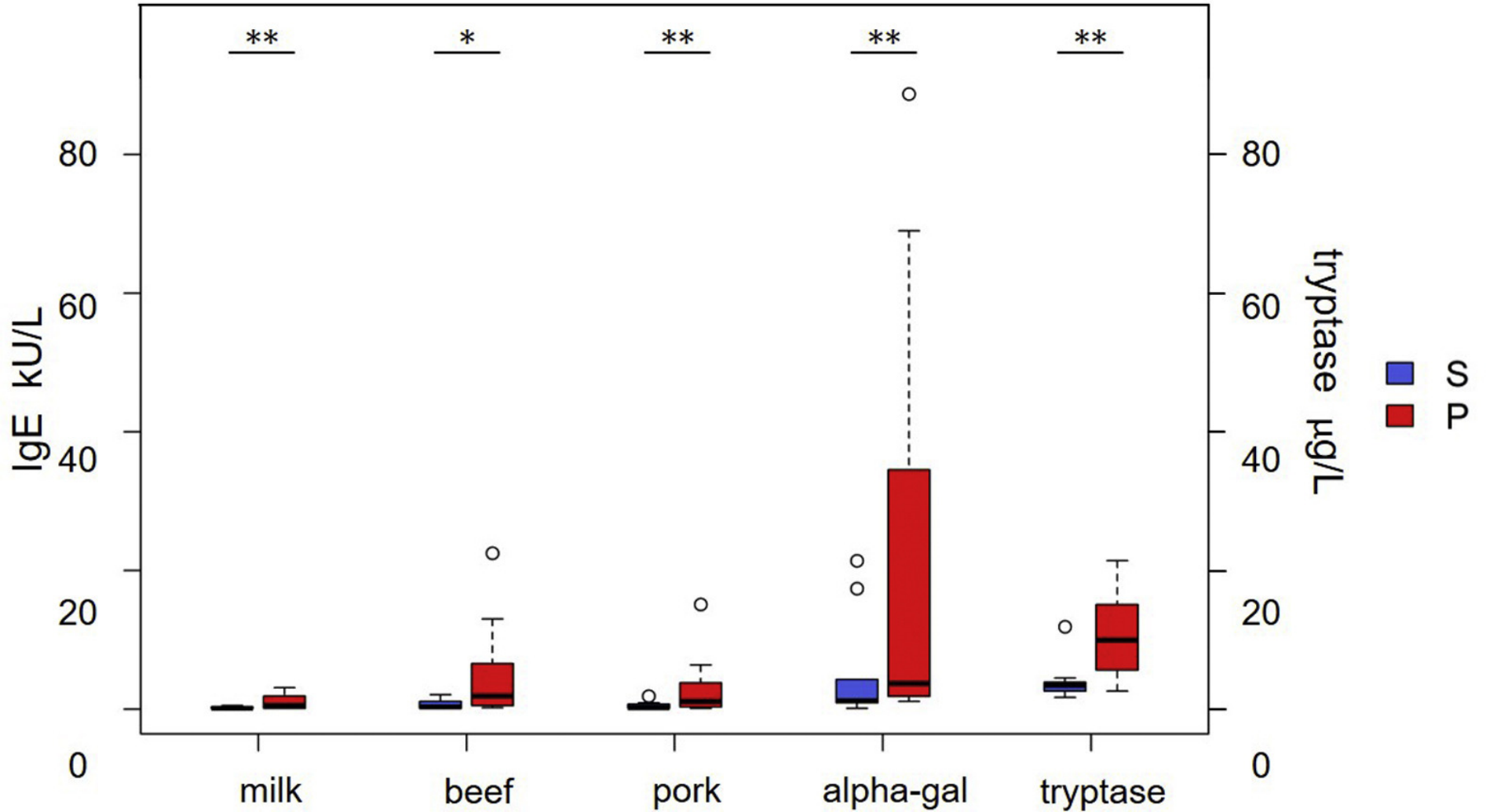
Online Repository / Supplement

Legends of figures

Fig. E1: SIgE and tryptase levels in patients with alpha-gal syndrome (P) and alpha-gal sensitized individuals (S). *p<0.05 and **p<0.01 for the comparison between groups using the Mann-Whitney U test. ○ individual outliers below the first quartile and above the third quartile.

Fig. E2: CD-sens and EC50 by alpha-Gal-HSA (Dextra Laboratories) and alpha-GAL (BÜHLMANN Laboratories) in patients with alpha-gal syndrome (P) and alpha-gal sensitized individuals (S). *p<0.05 for the comparison between groups using the Mann-Whitney U test. individual outliers below the first quartile and above the third quartile.

Fig. E3: Recombinant production and characterization of alpha-Gal-AHSG and AHSG. SDS-PAGE and Western blot analyses of recombinant AHSG and alpha-Gal-AHSG visualized either by Coomassie blue staining, anti-V5 epitope antibody (Thermo Fisher Scientific, Schwerte, Germany) or monoclonal anti-alpha-gal antibody (M86, Enzo Life Sciences, Farmingdale, USA).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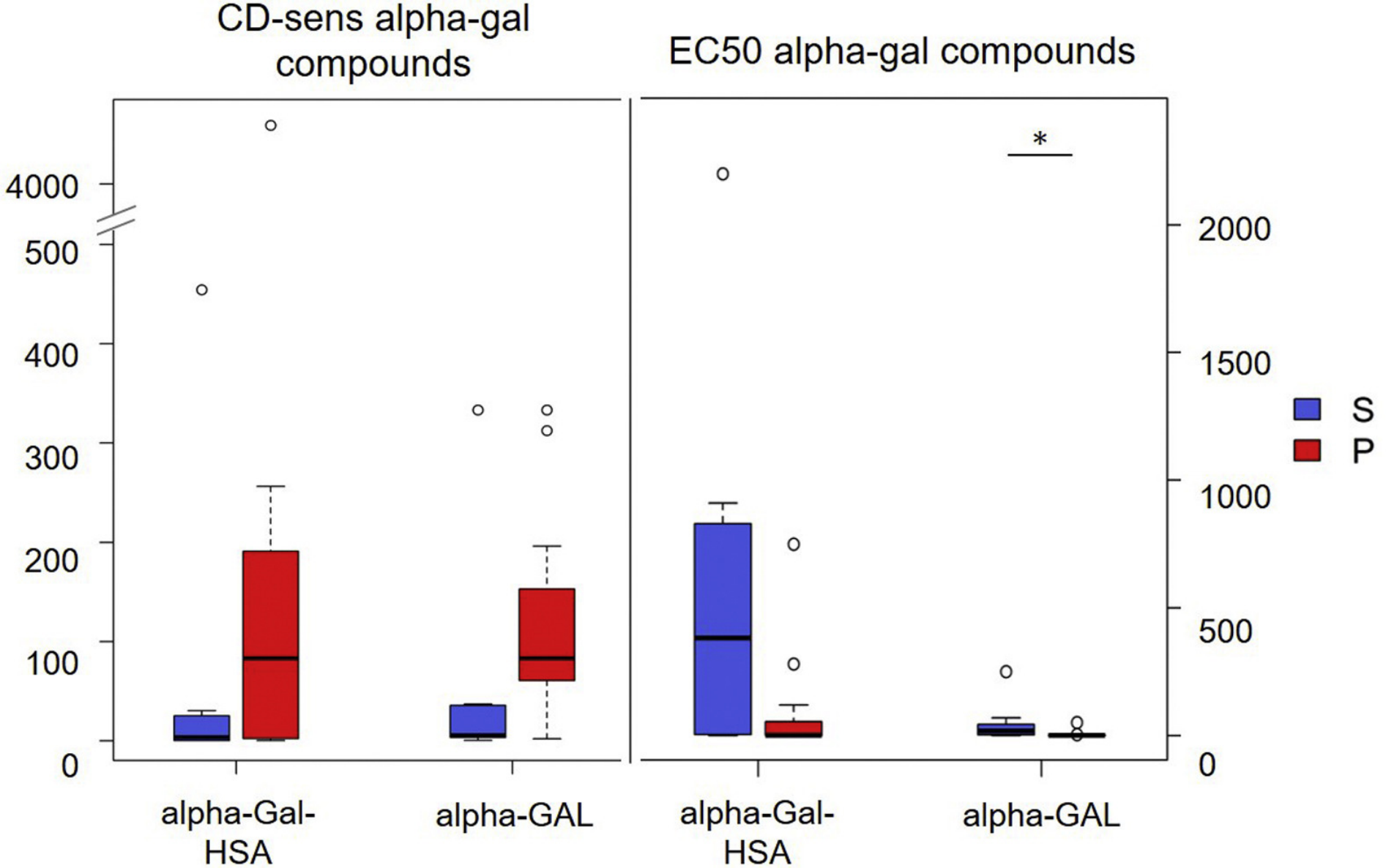
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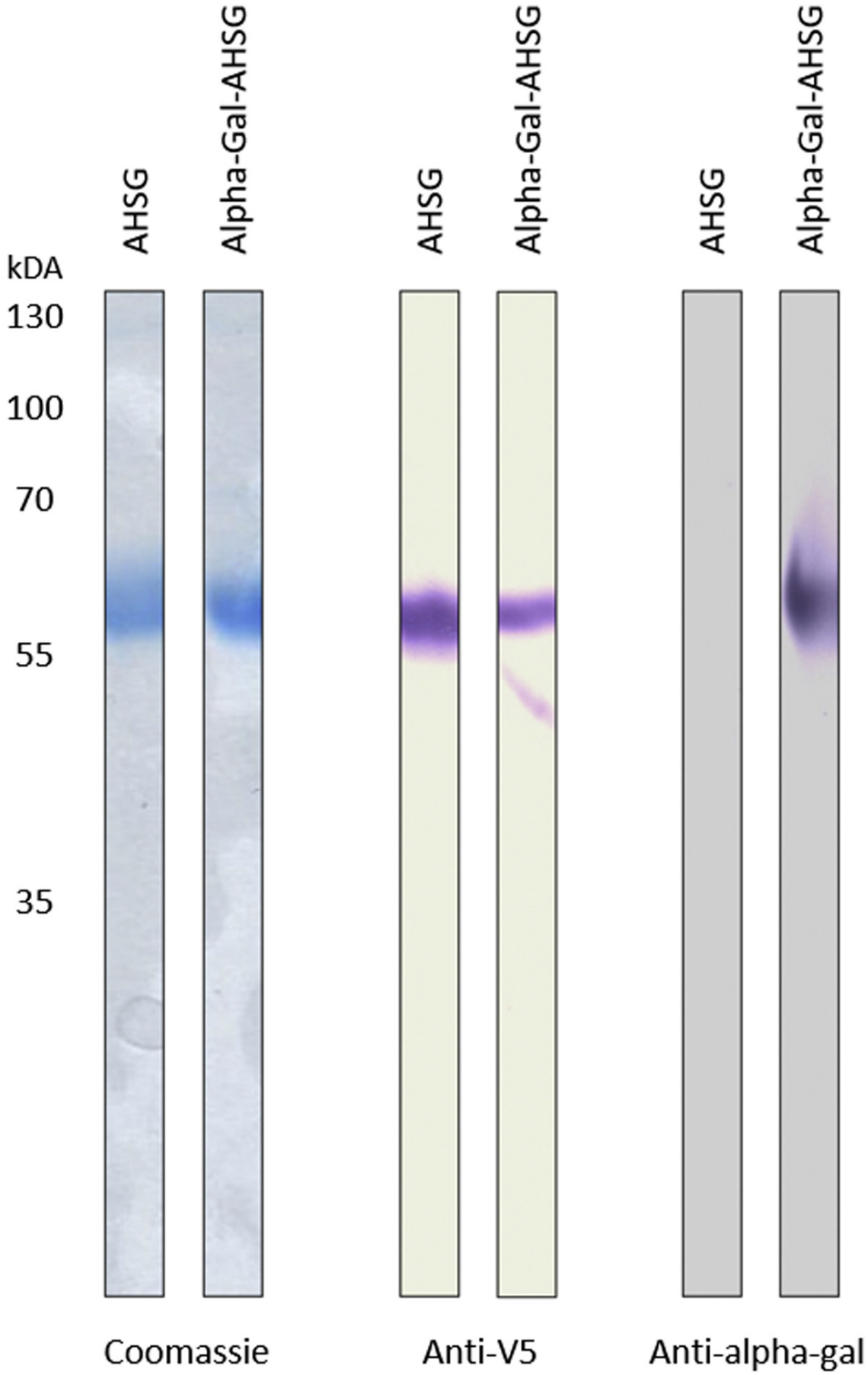
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Table E1 Allergic reaction (to pork kidney, pork or beef) severity grade classification, total IgE, tryptase, serum specific IgE of all study participants

				Serum specific IgE											
	Group	Severity grade classification (Ring & Messmer)	Total IgE	Tryptase	sIgE alpha-gal (o215)	sIgE pork (f26)	sIgE beef (f27)	sIgE Fel d 2 recom. cat (e220)	sIgE cow's milk (f2)	sIgE grass (g6)	sIgE birch (t3)	sIgE BetV1 (t215)	sIgE D. pter. (D1)	sIgE cat (e1)
			kU/L	kU/L	kU/L	kU/L	kU/L	kU/L	kU/L	kU/L	kU/L	kU/L	kU/L	kU/L
M01	C0	0	11.6	4.08	0.00	0.02	0.01	0.00	0.02	0.01	0.00	0.01	0.01	0.00
M02	C0	0	15.6	2.48	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00
M03	C0	0	24.3	4.62	0.01	0.00	0.01	0.00	0.02	0.00	0.00	0.00	0.01	0.00
M04	C0	0	15.4	4.02	0.00	0.00	0.02	0.00	0.01	0.00	0.00	0.00	0.01	0.00
M10	C0	0	35.2	4.58	0.00	0.01	0.01	0.00	0.03	0.00	0.00	0.00	0.01	0.00
M22	C0	0	17.1	4.79	0.00	0.26	0.19	0.00	0.10	0.01	0.00	0.00	0.01	0.00
M30	C0	0	58.5	7.63	0.00	0.00	0.03	0.00	0.00	0.02	0.00	0.00	0.02	0.00
M31	C0	0	60.9	3.00	0.01	0.00	0.02	0.00	0.07	0.00	0.00	0.00	0.42	0.00
		Median	17.1	4.08	0.00	0.00	0.02	0.00	0.02	0.00	0.00	0.00	0.01	0.00
		Min	27.3	3.00	0.00	0.00	0.01	0.00	0.02	0.04	0.02	0.00	0.02	0.01
		Max	680	5.97	0.12	0.07	0.09	0.11	0.12	51.9	88.3	100	49.0	2.07
L09	CA	0	533.0	*n.d.*	<0.1	*n.d.*	*n.d.*	<0.1	*n.d.*	1.80	0.00	0.09	49.00	0.00
L10	CA	0	123.0	*n.d.*	<0.1	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0.09	0.00	0.09	2.59	0.00
M05	CA	0	239.0	3.23	0.00	0.03	0.04	0.00	0.02	51.90	1.28	0.01	0.19	0.05
M06	CA	0	278.0	4.90	0.00	0.01	0.01	0.05	0.05	0.04	0.02	0.00	0.12	0.91
M07	CA	0	107.0	5.97	0.00	0.01	0.02	0.00	0.04	2.14	3.66	3.64	0.02	0.99
M08	CA	0	529.0	4.69	0.04	0.02	0.03	0.00	0.02	49.80	53.80	50.30	0.12	2.07
M09	CA	0	94.1	3.68	0.03	0.02	0.09	0.00	0.05	6.79	4.66	3.81	0.04	0.21
M11	CA	0	119.0	3.00	0.01	0.01	0.03	0.00	0.03	1.41	24.20	27.20	0.70	0.01
M25	CA	0	27.3	4.98	0.00	0.00	0.01	0.00	0.03	0.75	2.65	3.18	2.59	0.99
M27	CA	0	680.0	3.93	0.04	0.07	0.07	0.11	0.12	20.00	88.30	100.00	0.86	1.81
		Median	181	4.31	0.02	0.02	0.03	0.00	0.04	1.97	4.16	3.41	0.45	0.95
		Min	11.6	2.48	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.01	0.00
		Max	60.9	7.63	0.01	0.26	0.19	0.00	0.1	0.02	0.00	0.01	0.42	0.00
F009	P	2	34.3	17.00	1.13	0.53	0.66	0.00	0.44	0.00	0.00	0.00	0.05	0.34
F011	P	3	82.8	9.73	1.40	0.05	0.20	0.00	0.06	0.01	0.01	0.00	0.01	0.01
F013	P	3	395.0	5.46	>100	9.84	26.00	0.00	12.20	0.01	0.01	0.03	0.04	3.98
F015	P	2	46.0	5.58	2.41	0.21	0.48	0.00	0.05	0.00	0.00	0.00	0.01	0.02
F016	P	2	1504.0	10.20	88.70	3.39	9.78	0.08	2.18	5.43	2.21	0.08	3.56	1.22
F054	P	1	672.0	15.10	51.10	2.45	6.56	0.00	1.65	0.02	0.02	0.00	0.83	0.47
F070	P	2	61.3	2.48	19.10	2.28	4.24	0.00	0.84	0.00	0.00	0.00	0.02	0.26
F072	P	1	64.6	4.14	3.67	0.80	1.23	0.00	0.52	0.29	0.20	0.01	0.02	0.37
L02	P	3	765.0	1.73	69.00	6.36	13.00	<0.1	2.99	2.31	0.17	0.00	0.13	1.17
L03	P	1	80.0	10.20	5.52	9.00	11.00	0.00	1.80	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L04	P	2	72.0	*n.d.*	1.55	3.78	5.11	*n.d.*	1.93	0.00	0.00	0.00	27.00	0.00
L05	P	2	18.0	*n.d.*	2.02	0.73	0.50	<0.1	0.27	0.00	0.00	0.00	0.00	0.10
L06	P	2	32.0	*n.d.*	18.00	5.37	6.50	*n.d.*	1.37	27.00	0.00	0.09	0.00	0.00
L07	P	2	126.0	*n.d.*	12.00	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0.00	0.00	41.00	2.81	0.00
L11	P	1	143.0	*n.d.*	13.00	1.44	2.61	<0.1	*n.d.*	0.12	0.34	0.00	0.62	0.00
M12	P	2	120.0	10.80	15.50	1.05	1.30	0.00	0.24	0.00	0.00	0.00	0.01	0.07
M21	P	3	110.0	5.63	20.70	4.26	6.12	0.00	1.65	0.08	0.06	0.02	0.03	0.55
M23	P	2	53.5	2.45	13.80	3.01	4.40	0.00	0.89	0.02	0.01	0.00	0.01	0.84
M28	P	2	245.0	7.37	57.30	15.10	22.50	0.00	3.05	0.15	0.02	0.02	0.06	1.25

M32	P	3	86.4	2.63	3.20	0.25	0.60	0.00	0.14	0.00	0.00	0.00	0.01	0.03
M35	P	3	20.6	21.40	1.71	0.28	0.54	0.00	0.10	0.00	0.00	0.00	0.00	0.02
		Median	82.8	9.97	3.67	1.12	1.92	0.00	0.52	0.07	0.02	0.00	0.06	0.34
		Min	18.00	2.63	1.13	0.05	0.20	0.00	0.05	0.00	0.00	0.00	0.00	0.01
		Max	1504	21.4	88.7	15.1	22.5	0.09	3.05	27.0	2.21	41.0	27.0	1.25

L01	S	0	199.0	4.46	1.59	<0.1	<0.1	<0.1	<0.1	0.09	0.09	0.00	0.09	0.00
L08	S	0	168.0	*n.d.*	1.20	0.86	2.26	*n.d.*	0.16	0.00	0.00	0.00	0.00	0.00
L12	S	0	42.0	*n.d.*	0.96	0.15	0.33	<0.1	<0.1	14	*n.d.*	0.09	*n.d.*	*n.d.*
M13	S	0	28.7	6.28	2.13	0.03	0.03	0.00	0.03	0.06	0.01	0.01	0.03	0.01
M15	S	0	2254.0	11.90	0.11	0.10	0.12	0.07	0.09	10.20	0.31	0.06	0.47	3.41
M16	S	0	448.0	3.94	0.16	0.04	0.06	0.01	0.06	0.10	0.06	0.01	0.10	0.01
M17	S	0	33.8	3.04	0.86	0.05	0.15	0.07	0.04	0.02	0.01	0.01	0.14	0.02
M18	S	0	1080.0	2.55	21.40	0.72	1.85	0.11	0.24	45.60	66.90	75.70	15.20	0.00
M19	S	0	84.4	3.48	0.88	0.16	0.25	0.00	0.04	0.08	0.06	0.01	0.02	0.03
M20	S	0	74.5	3.44	4.34	0.51	1.08	0.00	0.27	0.03	0.03	0.01	0.05	0.11
M29	S	0	1466.0	2.34	17.40	0.86	2.07	0.03	0.52	0.10	0.06	0.10	0.12	0.42
M36	S	0	327.0	4.47	1.39	1.89	0.30	2.88	0.27	0.35	2.06	2.51	0.13	29.20
		Median	263	3.44	1.18	0.16	0.28	0.07	0.09	0.1	0.06	0.06	0.12	0.07
		Min	33.8	2.34	0.16	0.04	0.06	0.00	0.04	0.02	0.01	0.01	0.02	0.00
		Max	1466	4.46	21.4	1.89	2.07	2.88	0.52	45.6	66.9	75.7	15.2	29.2

Table E2 Results of skin prick tests, skin prick-to-prick tests and intracutaneous tests (Ict) of all study participants

		Skin prick test									Skin prick to prick test													Ict
	Group	Positive (Histamine)	Negative (NaCl)	Pork	Beef	Milk	Grass	Birch	D. pter.	Cat	Pork kidney (raw)	Pork kidney (cooked)	Pork (raw)	Pork (cooked)	Beef kidney (raw)	Beef kidney (cooked)	Beef (raw)	Beef (cooked)	Gelatin pork	Gelatin bovine	Cow's milk	Cetuximab (500 µg/mL)	Gelafundin (4%)	Gelafundin (4%)
M01	C0	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M02	C0	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M03	C0	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M04	C0	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M10	C0	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	neg	neg	*n.d.*
M22	C0	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
M30	C0	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
M31	C0	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
L09	CA	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	*n.d.*	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*
L10	CA	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	*n.d.*	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*
M05	CA	pos	neg	neg	neg	pos	pos	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M06	CA	pos	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M07	CA	pos	neg	neg	neg	neg	pos	pos	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M08	CA	pos	neg	neg	neg	pos	pos	pos	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M09	CA	pos	neg	neg	neg	pos	pos	pos	neg	pos	neg	neg	neg	pos	pos	pos	neg	neg	neg	*n.d.*	neg	neg	neg	*n.d.*
M11	CA	pos	neg	neg	neg	neg	pos	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	*n.d.*
M25	CA	pos	neg	neg	neg	neg	pos	pos	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*
M27	CA	pos	neg	neg	neg	neg	pos	pos	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*
F009	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	pos	pos	neg	pos	neg	neg	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos
F011	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	pos	pos	neg	pos	neg	neg	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos
F013	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	pos	pos	neg	pos	pos	neg	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos
F015	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	pos	neg	neg	pos	pos	neg	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos
F016	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	pos	pos	neg	pos	pos	neg	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos
F054	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	pos	neg	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos
F070	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*pos*	*pos*	*pos*	*neg*	pos	pos	neg	pos	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos
F072	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*pos*	*pos*	*neg*	*neg*	pos	pos	neg	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos
L02	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*pos*	*n.d.*	*pos*	*n.d.*	n.d.	n.d.	neg	n.d.	*n.d.*	*n.d.*	*neg*	*n.d.*	*n.d.*	neg

L03	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	neg	pos	*n.d.*	*n.d.*	pos	pos	*n.d.*	*n.d.*	pos	*n.d.*	neg	pos
L04	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	neg	neg	*n.d.*	*n.d.*	pos	*n.d.*	*n.d.*	*n.d.*	neg	pos	neg	pos
L05	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	pos	*n.d.*	*n.d.*	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	neg	neg	neg	pos
L06	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	*n.d.*	*n.d.*	neg	pos	neg	pos
L07	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	pos	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	*n.d.*	*n.d.*	neg	pos	neg	pos
L11	P	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	neg	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	neg	neg	neg	pos
M12	P	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
M21	P	pos	neg	pos	neg	neg	neg	neg	neg	neg	pos	pos	pos	neg	pos	pos	pos	neg	neg	*n.d.*	pos	neg	neg	*n.d.*
M23	P	pos	neg	pos	neg	neg	neg	neg	neg	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	*n.d.*
M28	P	pos	neg	pos	pos	neg	neg	neg	neg	neg	pos	pos	pos	pos	pos	pos	neg	pos	pos	pos	pos	pos	neg	*n.d.*
M32	P	pos	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos	neg	neg	pos	pos	neg	neg	neg	neg	neg	pos	neg	*n.d.*
M35	P	pos	neg	neg	*n.d.*	pos	neg	neg	neg	pos	pos	neg	pos	neg	pos	pos	pos	pos	pos	pos	neg	pos	neg	*n.d.*
L01	S	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	neg	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	neg	*n.d.*	neg	neg
L08	S	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	neg	*n.d.*	*n.d.*	*n.d.*	neg	neg	*n.d.*	*n.d.*	neg	neg	neg	neg
L12	S	pos	neg	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	pos	*n.d.*	neg	neg	*n.d.*	*n.d.*	pos	neg	*n.d.*	*n.d.*	pos	neg	neg	pos
M13	S	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M15	S	pos	neg	neg	neg	neg	pos	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M16	S	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M17	S	pos	neg	neg	*n.d.*	neg	neg	neg	neg	neg	pos	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M18	S	pos	neg	neg	*n.d.*	pos	pos	pos	pos	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	*n.d.*
M19	S	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	*n.d.*	neg	neg	neg	neg
M20	S	pos	neg	neg	pos	neg	neg	neg	neg	neg	pos	pos	neg	neg	pos	neg	neg	neg	neg	*n.d.*	neg	neg	neg	*n.d.*
M29	S	pos	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos	pos	neg	pos	pos	neg	neg	neg	neg	neg	pos	neg	*n.d.*
M36	S	pos	neg	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	pos	neg	neg	neg	neg	neg	neg	*n.d.*

Table E3 Results of the basophil activation test (%CD63^{+}) of all study participants

					Alpha-GAL[a]						Pork kidney extract						Alpha-Gal-HSA[b]						Alpha-Gal-AHSG	Pork[a]			Beef[a]		
	Group	Baseline	Positive controle anti-FcεRI	Positive controle fMLP	1000 (ng/mL)	200 (ng/mL)	40 (ng/mL)	8 (ng/mL)	1.6 (ng/mL)	0.32 (ng/mL)	22.7 (µg/mL)	2.27 (µg/mL)	0.227 (µg/mL)	0.0227 (µg/mL)	0.00227 (µg/mL)	0.00022 (µg/mL)	2272 (ng/mL)	227 (ng/mL)	22.7 (ng/mL)	2.27 (ng/mL)	0.22 (ng/mL)	0.022 (ng/mL)	227.3 (ng/mL)	22.7 (ng/mL)	4.5 (ng/mL)	0.9 (ng/mL)	22.7 (ng/mL)	4.5 (ng/mL)	0.9 (ng/mL)
M01	C0	0,00	39,92	1,02	0,65	0,00	0,44	0,00	0,00	0,00	0,21	0,40	0,20	0,26	0,27	0,30	0,63	0,00	0,30	0,32	0,00	0,75	1,22	0,00	1,67	1,17	0,56	0,97	1,00
M02	C0	0,61	81,82	56,31	0,21	1,15	0,98	2,40	0,80	0,26	0,41	0,00	0,20	0,63	0,60	0,27	0,27	1,15	0,61	0,67	0,55	1,47	2,16	1,03	2,82	0,88	0,93	1,55	1,05
M03	C0	0,00	68,11	38,97	0,00	0,00	0,00	0,00	0,00	0,00	0,21	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
M04	C0	0,00	81,40	26,68	0,00	0,20	0,00	0,00	0,00	0,61	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,41	0,00	0,00	0,00	0,00	0,20	0,28
M10	C0	0,21	0,98	5,31	0,58	0,58	0,94	0,31	0,00	0,56	3,01	1,23	0,42	0,99	0,64	0,63	1,66	0,28	0,22	1,01	0,34	0,30	0,97	0,52	0,42	0,32	0,92	0,32	0,67
M22	C0	0,21	90,12	81,06	0,18	0,00	0,58	0,26	0,19	0,60	0,21	0,00	0,00	0,00	0,21	0,00	0,00	0,00	0,20	0,27	0,00	0,38	0,00	0,00	0,00	0,00	0,00	0,41	0,40
M30	C0	0,21	83,62	15,10	0,22	0,00	0,00	0,00	0,00	0,41	0,41	0,41	0,20	0,61	0,20	0,30	0,20	0,41	0,27	0,00	0,20	0,00	0,31	0,00	0,00	0,32	0,20	0,00	0,00
M31	C0	0,41	82,74	20,16	0,00	0,23	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,56	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
L09	CA	0,60	53,73	47,01	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0,60	0,40	0,40	0,00	0,39	0,20	0,39	0,20	0,00	0,00	0,00	0,00	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L10	CA	0,45	87,40	15,33	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	0,43	1,90	0,41	0,60	1,83	1,17	0,21	1,36	0,22	0,81	0,79	0,36	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
M05	CA	0,00	66,33	44,40	0,00	0,21	0,00	0,52	0,00	0,21	0,00	0,00	0,00	0,29	0,00	0,00	0,00	0,23	0,00	0,41	0,00	0,40	0,00	0,20	0,20	0,20	0,20	0,00	0,40
M06	CA	0,21	10,99	4,50	1,05	0,83	2,20	1,01	1,01	1,83	0,67	1,66	1,71	2,02	1,85	1,02	0,00	1,38	2,53	3,05	0,41	1,98	0,60	0,83	0,81	2,16	1,41	2,26	1,83
M07	CA	0,20	24,25	26,86	0,00	0,68	0,65	0,68	0,00	0,00	0,56	0,47	0,39	0,78	0,28	0,43	0,00	0,00	0,00	0,33	0,00	0,00	0,33	0,34	0,33	0,68	0,00	0,00	0,36
M08	CA	0,20	85,29	10,52	0,21	0,24	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,42	0,00	0,00	0,20	0,31	0,00	0,00	0,00	0,00	0,00	0,31	0,21	0,00	0,40	0,24	0,00
M09	CA	0,66	93,63	54,14	0,00	0,61	0,62	0,41	0,20	0,41	2,61	0,43	0,61	0,41	0,41	1,04	4,85	0,42	0,51	1,12	0,85	0,61	0,00	0,20	0,41	0,41	0,20	0,42	0,21
M11	CA	0,20	22,42	15,16	0,00	0,00	0,21	0,44	0,61	0,00	0,00	0,15	0,00	0,00	0,39	0,00	0,00	0,00	0,00	0,00	0,20	0,00	0,20	0,20	0,00	0,41	0,62	0,00	0,21
M25	CA	0,00	81,51	56,16	0,00	0,00	0,00	0,22	0,58	0,31	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,20	0,00	0,85	0,40	0,20	0,29	0,00	0,67	0,00
M27	CA	0,20	36,44	10,10	0,20	0,00	0,00	0,20	0,00	0,41	0,80	0,00	0,40	0,00	0,00	0,35	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
F009	P	2,17	43,71	13,80	37,01	41,41	46,95	47,51	48,09	43,78	33,33	20,71	19,55	4,88	3,04	4,38	35,29	41,53	43,88	52,19	46,52	33,27	22,11	2,02	2,50	1,62	3,23	2,25	2,97
F011	P	0,62	86,51	26,12	30,07	58,89	38,25	51,66	55,17	78,27	30,54	42,77	69,18	75,30	38,89	36,69	71,26	13,49	0,60	0,00	0,20	0,20	12,44	0,61	1,95	0,89	0,29	0,20	1,21
F013	P	10,71	59,72	61,85	17,46	23,64	48,94	63,28	47,12	44,12	45,18	56,91	26,87	12,32	13,50	17,48	20,59	27,10	53,77	65,08	57,02	9,59	64,81	15,37	12,24	11,36	11,86	6,98	15,57
F015	P	3,12	86,27	44,19	24,41	45,31	29,62	30,45	42,30	76,37	35,37	26,57	47,96	94,85	88,08	92,37	91,29	64,54	8,61	1,98	3,38	2,89	91,40	4,64	4,42	4,39	5,40	6,49	4,82
F016	P	1,22	22,94	51,12	7,26	3,24	6,53	23,34	17,51	8,30	24,35	33,87	1,81	1,01	0,40	0,00	8,94	4,26	8,72	27,68	6,68	1,62	37,02	0,20	0,40	0,00	0,40	0,60	0,61
F054	P	7,31	71,81	35,27	60,08	72,56	79,75	85,08	52,33	37,92	80,71	52,14	7,60	6,88	7,97	7,30	61,02	74,69	81,89	73,16	29,71	13,40	60,33	4,65	6,52	5,34	6,71	8,12	8,70
F070	P	23,99	15,54	48,91	16,64	10,27	11,07	10,08	10,86	14,79	23,49	13,10	11,11	25,15	35,79	34,15	19,80	34,22	27,68	18,00	15,54	17,59	30,74	24,14	27,64	24,90	20,40	19,76	23,77
F072	P	2,51	27,09	38,46	14,80	17,34	8,79	14,51	18,66	22,24	14,21	24,02	24,34	48,16	48,98	44,13	38,56	39,60	5,98	3,18	1,63	0,54	47,64	3,27	2,26	3,97	2,44	6,16	2,70
L02	P	3,10	67,30	26,40	19,08	16,84	56,34	67,54	44,76	13,65	64,65	49,69	6,74	1,43	0,84	0,43	52,48	62,35	41,30	4,86	0,80	0,60	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*

L03	P	2,04	10,93	33,60	10,02	7,46	6,20	12,53	11,09	5,23	17,03	23,08	5,84	2,62	2,41	1,00	19,27	14,46	11,13	3,60	2,23	1,00	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L04	P	4,01	91,60	66,87	42,97	40,32	47,32	70,02	71,12	39,24	64,38	68,99	24,85	3,79	13,57	1,20	85,91	72,64	57,11	10,57	1,81	0,40	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L05	P	3,68	84,05	16,28	11,86	30,54	69,50	58,39	22,29	8,78	83,50	82,12	19,05	4,76	2,47	9,73	81,36	59,51	13,07	50,20	10,27	2,71	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L06	P	4,56	26,42	13,11	38,27	39,79	49,16	57,00	31,58	6,46	46,64	66,06	17,63	21,49	5,42	22,20	35,01	37,89	18,97	47,22	30,42	19,39	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L07	P	0,00	24,07	12,92	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	27,36	3,53	0,21	0,20	0,00	0,42	28,99	11,80	0,60	0,00	0,20	0,00	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L11	P	0,41	80,80	33,00	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	82,70	55,42	0,19	0,20	0,20	0,41	72,35	37,72	2,61	0,00	0,39	0,60	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
M12	P	0,66	0,45	1,34	0,22	0,65	1,06	0,43	0,00	0,00	0,46	0,45	0,44	0,22	0,44	0,21	0,00	0,22	0,00	0,44	0,43	0,65	0,91	0,00	0,42	0,44	0,22	0,00	0,21
M21	P	0,44	1,20	42,08	0,79	0,00	0,63	0,98	0,21	0,68	1,22	0,80	0,60	0,40	0,40	1,08	1,41	0,55	0,95	0,28	1,46	1,14	0,65	0,27	0,68	0,32	0,00	0,31	0,63
M23	P	24,06	21,74	91,37	17,46	11,88	29,84	46,95	69,73	66,76	21,14	43,95	51,89	41,40	33,66	40,74	16,06	10,93	28,85	56,52	51,46	36,98	59,63	50,45	44,56	51,37	56,70	48,55	48,15
M28	P	3,12	12,36	48,77	7,59	4,76	13,85	19,21	0,00	0,21	12,95	22,78	10,98	5,43	5,13	4,13	11,92	4,83	20,97	5,72	8,52	4,80	16,08	0,67	2,77	2,30	1,83	4,11	3,48
M32	P	0,88	87,20	2,66	47,63	38,17	64,74	73,80	46,28	25,55	76,51	68,89	6,60	0,00	0,00	0,20	84,55	40,76	69,20	76,05	33,75	9,07	36,52	1,03	0,00	1,33	0,62	0,21	0,00
M35	P	4,63	68,32	84,08	28,63	25,60	30,49	65,93	71,43	46,71	75,37	72,42	18,44	10,10	7,55	5,31	41,31	29,19	30,63	68,76	22,22	8,21	76,57	7,99	10,62	6,83	6,02	5,76	6,56
L01	S	0,80	36,84	17,43	1,41	1,99	1,19	*n.d.*	*n.d.*	*n.d.*	2,25	0,61	0,80	0,40	0,40	*n.d.*	2,20	0,40	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L08	S	0,00	73,02	21,28	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	1,68	0,20	0,00	0,00	0,00	0,00	4,20	0,20	0,00	0,00	0,00	0,20	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
L12	S	0,00	74,70	33,70	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	35,92	15,61	0,20	0,36	0,60	0,00	9,68	6,84	1,62	0,40	0,20	0,41	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*
M13	S	0,67	5,37	20,28	0,34	0,98	0,31	0,90	0,32	0,00	0,43	0,29	0,31	0,00	0,85	1,25	1,37	0,61	1,64	0,00	1,41	0,95	0,28	1,31	1,04	0,65	0,84	0,32	0,00
M15	S	0,28	34,43	21,54	0,31	0,69	0,00	0,32	0,00	0,00	0,31	0,81	0,00	0,00	0,21	0,00	0,34	0,34	0,34	0,00	0,67	0,33	0,00	0,33	1,31	1,03	0,00	0,00	0,00
M16	S	0,00	84,62	66,84	0,20	0,20	0,50	0,49	0,00	0,00	0,33	0,21	0,00	0,60	0,27	0,00	0,00	0,26	0,00	0,00	0,28	0,28	0,00	0,00	0,64	0,00	0,00	0,27	0,00
M17	S	0,40	72,32	18,88	16,06	39,03	38,41	6,63	0,41	0,00	30,56	0,58	0,20	0,30	0,20	0,00	46,96	2,85	0,20	0,20	0,27	0,00	21,26	0,00	0,00	0,00	0,00	0,30	0,00
M18	S	1,02	65,54	16,76	19,23	9,04	1,77	0,60	0,20	0,40	4,61	0,20	0,00	0,00	0,00	0,00	4,35	0,00	0,00	0,00	0,00	0,20	37,24	0,20	0,00	0,00	0,20	0,00	0,00
M19	S	0,00	39,45	49,14	17,34	23,32	17,83	4,58	0,18	0,21	23,33	0,21	0,00	0,00	0,00	0,21	16,49	23,76	14,14	2,69	0,21	0,42	5,03	0,00	0,00	0,00	0,21	0,21	0,21
M20	S	1,20	54,78	30,26	14,69	19,60	47,26	47,73	13,29	2,99	47,68	46,77	5,50	2,53	1,12	0,21	12,50	20,04	51,46	21,34	3,06	2,24	44,51	0,24	0,31	1,07	0,84	0,93	0,68
M29	S	0,00	63,24	23,85	53,73	51,35	66,67	56,44	25,00	7,63	69,01	22,67	0,00	0,00	0,00	0,00	52,70	47,55	68,94	15,98	1,11	1,49	30,92	0,50	0,00	0,00	0,00	0,75	0,00
M36	S	0,41	86,15	52,24	20,20	12,14	20,93	26,11	18,59	16,70	30,49	10,89	1,62	0,81	0,40	1,01	30,04	14,23	19,68	22,98	13,96	17,92	10,55	0,00	0,20	0,60	0,61	0,21	0,00

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