birth. The severity of maternal allergic disease might play a more important role in SGA than maternal sensitization itself. Our results highlighted the comorbidity of maternal allergic disorders and SGA offspring and suggested that whether or not IUGR occurs, and greater attention needs to be paid to this association when following pregnant women with AD and asthma in a clinical setting.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interests.

ORCID

K. Yamamoto-Hanada http://orcid.org/0000-0003-1288-9620
K. Ishitsuka http://orcid.org/0000-0002-2944-7012
M. Konishi http://orcid.org/0000-0002-1255-3644
L. Yang http://orcid.org/0000-0002-3936-6371
H. Saito http://orcid.org/0000-0002-6630-8337
Y. Ohya http://orcid.org/0000-0002-1560-3942

M. Saito[1]
K. Yamamoto-Hanada[1]
K. Pak[2]
T. Ayabe[1]
H. Mezawa[1]
K. Ishitsuka[1]
M. Konishi[1]
L. Yang[1]
K. Matsumoto[1]
H. Saito[1]
Y. Ohya[1]
The Japan Environment and Children's Study (JECS) Group
[1]*Medical Support Center for the Japan Environment and Children's Study, National Center for Child Health and Development, Tokyo, Japan*
[2]*Department of Clinical Medicine (Biostatistics), Kitasato University School of Pharmacy, Tokyo, Japan*
Email: yamamoto-k@ncchd.go.jp

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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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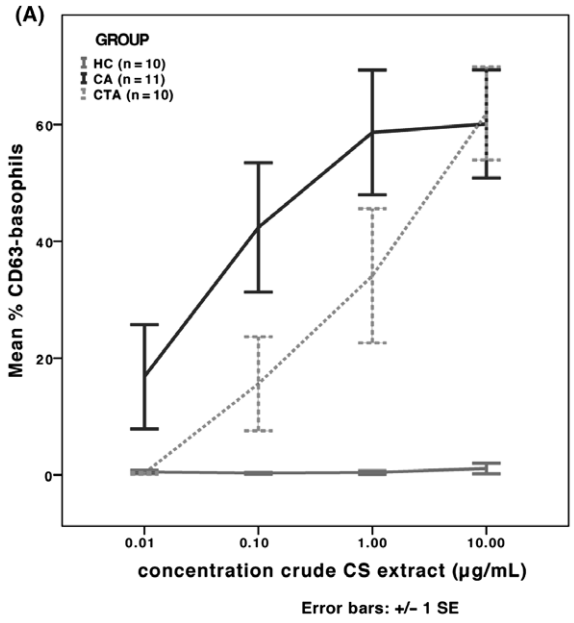
Cannabis allergy: A diagnostic challenge

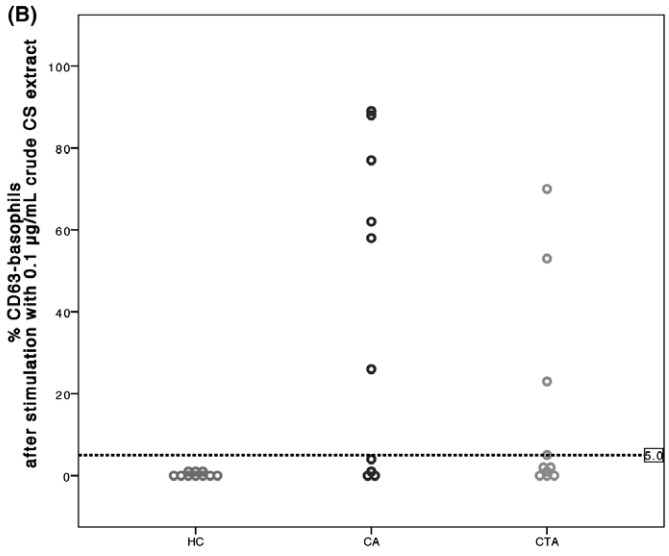
To the editor,

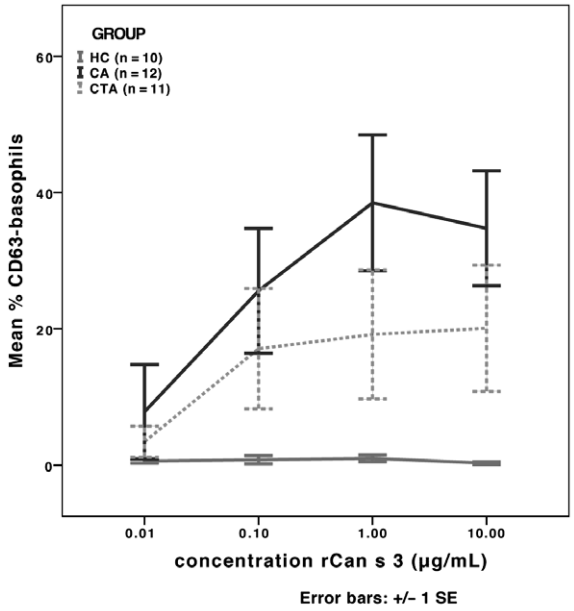
In the absence of a validated confirmatory test to document cannabis allergy, physicians use skin prick-prick testing with cannabis buds or leaves to confirm their clinical suspicion of a cannabis allergy.[1-4] Obviously, this approach is difficult to standardize as results are dependent on the source material. Our earlier study[5] revealed that a basophil activation test (BAT) with an nsLTP rich cannabis extract could reliably diagnose cannabis allergy but leaves room for improvement. Here, we take advantage of the newly expressed Can s 3 protein[6] to develop 3 flow cytometric diagnostics for cannabis allergy; a BAT with rCan s 3, a BAT with a crude CS extract, and a bead assay (CBA) quantifying sIgE to rCan s 3.

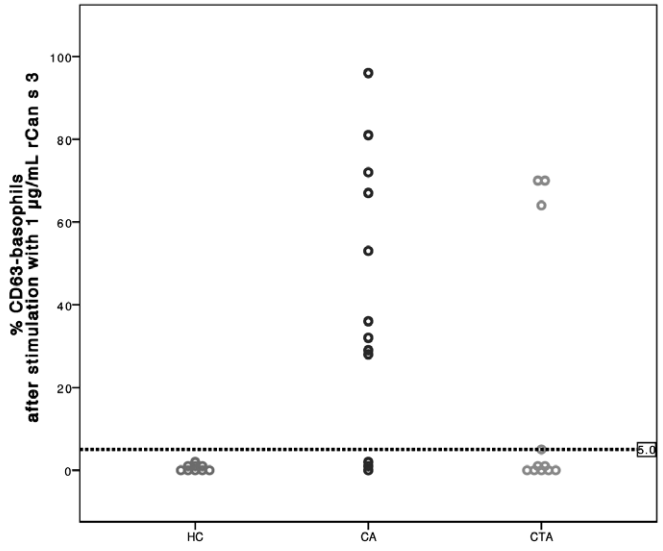
Twelve patients reporting immediate respiratory and cutaneous symptoms (as detailed in Table S2) on exposure to cannabis were

Abbreviations: BAT, basophil activation test; CA, cannabis allergic participants; CBA, cytometric bead array; CI, confidence interval; CS, *Cannabis sativa*; CTA, cannabis tolerant but atopic participants with pollen and LTP sensitizations; HC, healthy controls; NPV, negative predictive value; nsLTP, nonspecific lipid transfer protein; PPV, positive predictive value; rCan s 3, recombinant Can s 3 protein from *Cannabis sativa*; sIgE, specific immunoglobulin E.


(A)
GROUP
HC (n = 10)
CA (n = 11)
CTA (n = 10)
Mean % CD63-basophils
0
20
40
60
0.01
0.10
1.00
10.00
concentration crude CS extract (µg/mL)
Error bars: +/– 1 SE


(B)
% CD63-basophils
after stimulation with 0.1 µg/mL crude CS extract
0
20
40
60
80
100
5.0
HC
CA
CTA


GROUP
HC (n = 10)
CA (n = 12)
CTA (n = 11)
Mean % CD63-basophils
0
20
40
60
0.01
0.10
1.00
10.00
concentration rCan s 3 (µg/mL)
Error bars: +/– 1 SE


% CD63-basophils
after stimulation with 1 µg/mL rCan s 3
0
20
40
60
80
100
5.0
HC
CA
CTA

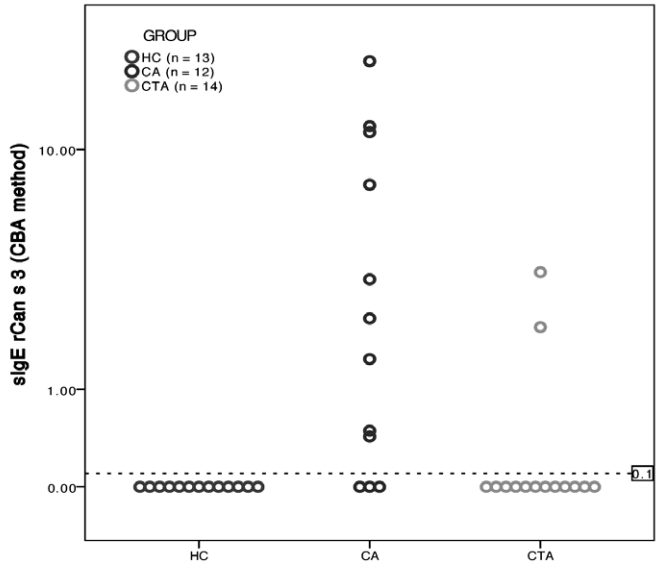

GROUP
HC (n = 13)
CA (n = 12)
CTA (n = 14)
sIgE rCan s 3 (CBA method)
0.00
1.00
10.00
0.1
HC
CA
CTA

FIGURE 1 A, Dose-response curve showing basophil activation after 10-1-0.1-0.01 μg/mL rCan s 3 and a crude cannabis extract. B, Basophil activation after 1 μg/mL rCan s 3 and 0.1 μg/mL crude cannabis extract and sIgE measurement for rCan s 3 expressed in kU_A/L. Basophil activation is expressed as net percentage of CD63 expressing basophils. HC, healthy controls; CA, cannabis allergic patients; CTA, cannabis tolerant but atopic patients (with pollen and nsLTP sensitizations); SE, standard error of the mean

selected (CA). Exposure to cannabis implied smoking or ingestion of cannabis, except one patient who experienced repetitive generalized respiratory and cutaneous symptoms upon passive cannabis smoke exposure.[7] Fifteen cannabis tolerant healthy controls (HC) and 14 cannabis tolerant atopic (CTA) patients, all demonstrating pollen and nsLTP sensitization, were also enrolled. All control subjects reported repeated smoking of cannabis in the last year without symptoms apart from its psychoactive effects. These study groups were included because the evaluation of diagnostics cannot be considered complete when it fails to identify conditions that might affect the outcome, for example multiple allergic sensitizations. Definitions of pollen and nsLTP sensitizations can be found in the online Supplementary Information. BATs were performed with 0.01, 0.1, 1 and 10 μg/mL of rCan s 3 (expressed as previously described[6]) and a crude cannabis extract (extraction method see Rudeschko[8]). Specific IgE to rCan s 3 was quantified using a CBA (BD Biosciences, Franklin Lakes, NJ, USA). Details of the methods are provided in the online Supplementary Information.

Ten of twelve CA patients have a pollen and nsLTP sensitization, one patient demonstrates a pollen but no nsLTP sensitization, and one patient is nsLTP-sensitized without a pollen sensitization. Demographic data, sensitization profiles and history are summarized in Tables S2 and S3.

Figure 1A displays a dose-response curve for the BAT with rCan s 3 and the cannabis extract and corresponds with the mathematical comparison of the areas under de curve (ROC analyses) showing that the 3 highest concentrations in both BATs are equally performant to discriminate HC from CA. The lowest allergen concentration showing optimal activation potential in the CA group and fewest clinically irrelevant results in the CTA group was chosen resulting in 0.1 μg/mL crude cannabis extract and 1 μg/mL rCan s 3 with a cut-off of >5% CD63+ basophils, corresponding to the highest activation percentage seen in HC and the test variability (4.5% for 500 gated basophils). Both BATs show an absolute specificity and a similar sensitivity (Table 1). The individual results for both BATs with the chosen concentrations are shown in Figure 1B. This figure also shows the individual results for the sIgE measurement of rCan s 3. ROC analysis shows a specificity of 100% and a sensitivity of 75% for a cut-off value of 0.10 kU_A/L (technical limit of detection for this technique).

These results show an absolute specificity and a sensitivity between 60% and 75% for all 3 tests, comparing HC and CA. Notably, the intertest differences for the sensitivity and specificity are not significant. When these results are compared to the initial exploration of the rCan s 3 allergen[6] which observed 30% sensitivity applying a streptavidin-ImmunoCAP method in patients mainly exhibiting respiratory symptoms, it appears test sensitivity and sensitization patterns depend on the clinical phenotype, as our participants with CA had both respiratory and cutaneous reactions. Obviously, differences in test performance and sensitization profiles might also result from the use of different techniques.

The validation of novel diagnostics cannot be considered complete when it fails to identify confounders that could affect the outcome of the evaluation. Therefore, the diagnostic test performance was additionally investigated in a group of individuals tolerant to cannabis but showing a pollen and nsLTP sensitization profile similar to the majority of our CA patients, the CTA group. From this "worst case scenario," it appears both BATs and the sIgE assay are reliable with only limited clinically irrelevant results in CTA. It is likely but currently unproven that these low numbers of clinically irrelevant Can s 3 results in the nsLTP-sensitized CTA group reflect genuine CA to result from Can s 3-specific epitopes that go undetected in more traditional nsLTP tests, mainly the rPru p 3 assay.

TABLE 1 Test Performance

		Healthy controls (HC) vs cannabis allergics (CA)	Cannabis tolerant atopics (CTA) vs cannabis allergics (CA)
BAT rCan s 3	SENSITIVITY (95% CI)	66.7% (34.9-90.1)	-
	SPECIFICITY (95% CI)	100.0% (69.2-100.0)	-
	PPV (95% CI)	100.0% (69.2-100.0)	72.7% (48.4-88.3)
	NPV (95% CI)	71.4% (52.9-84.8)	66.7% (45.4-82.8)
BAT crude CS extract	SENSITIVITY (95% CI)	60.0% (26.2-87.8)	-
	SPECIFICITY (95% CI)	100.0% (69.2-100.0)	-
	PPV (95% CI)	100.0% (69.2-100.0)	66.7% (40.2-85.6)
	NPV (95% CI)	71.4% (53.9-84.2)	66.7% (38.5-86.5)
sIgE rCan s 3	SENSITIVITY (95% CI)	75.0% (42.8-94.5)	-
	SPECIFICITY (95% CI)	100.0% (75.3-100.0)	-
	PPV (95% CI)	100.0% (75.3-100.0)	81.8% (54.5-94.4)
	NPV (95% CI)	81.3% (61.9-92.0)	80.0% (59.5-91.6)

CI, confidence interval; NPV, negative predictive value; PPV, positive predictive value.

As displayed in Figure S3, 3 CA patients show triple negative results. One possible explanation is the potential shortcoming of our crude cannabis extract that could lack sufficient amounts of other relevant allergens to trigger basophil responsiveness and sIgE binding. Therefore, further studies are warranted to identify and produce other allergens, as these could be applied to spike natural extracts or to compose mixtures of allergens. A second possibility is that some patients might experience symptoms to contaminants such as moulds that have been found in illicit cannabis plantations.[9] A third hypothesis is that the reported symptoms could be caused by non-IgE–mediated reactions on exposure to cannabis which cannot be demonstrated by our tests. This theory is fostered by the finding that both this study and Rihs et al[6] show patients with clear symptoms on cannabis exposure but no response in BAT or sIgE to rCan s 3 nor a crude extract.

In summary, BAT with a crude cannabis extract, rCan s 3 and sIgE (CBA) with rCan s 3 might be reliable diagnostics identifying two-thirds of our patients. However, different questions remain to be resolved including the identification and expression of novel allergens as these could benefit the tests. Furthermore, larger studies are mandatory to confirm our observations including the fact that sensitization profile and test performance might depend on the clinical phenotype.

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CONFLICT OF INTEREST

All authors have no conflict of interest to declare.

ORCID

I. I. Decuyper http://orcid.org/0000-0001-8127-5791
M. A. Faber http://orcid.org/0000-0002-1277-5052
H. P. Rihs http://orcid.org/0000-0002-8991-1526
A. L. Van Gasse http://orcid.org/0000-0003-1657-5135
M. M. Hagendorens http://orcid.org/0000-0001-6361-9503
V. Sabato http://orcid.org/0000-0002-1321-314X
L. De Clerck http://orcid.org/0000-0002-4622-6289
D. G. Ebo http://orcid.org/0000-0003-0672-7529

I. I. Decuyper[1,2]
M. A. Faber[1]
H. Lapeere[3]
C. Mertens[1]
H. P. Rihs[4]
A. L. Van Gasse[1]
M. M. Hagendorens[1,2]
V. Sabato[1]
C. H. Bridts[1]
L. De Clerck[1]
D. G. Ebo[1]

[1]*Immunology-Allergology-Rheumatology, University of Antwerp - University Hospital of Antwerp, Antwerp, Belgium*
[2]*Pediatrics, University of Antwerp - University Hospital of Antwerp, Antwerp, Belgium*
[3]*Dermatology, University of Ghent, Ghent University Hospital, Gent, Belgium*
[4]*IPA - Institute for Prevention and Occupational Medicine, German Social Accident Insurance, Ruhr-University Bochum, Bochum, Germany*
Email: immuno@uantwerpen.be

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SUPPORTING INFORMATION

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

Methods

Participants

Patients and controls were included through the outpatients' clinic of Allergology at the University Hospital of Antwerp and the Dermatology department of the University Hospital of Ghent, Belgium. The local ethics committee of both hospitals approved this study (B300201524055) and patients or their representatives approved an informed consent in accordance with the Declaration of Helsinki. Participants with chronic urticaria, mastocytosis or uncontrolled asthma were excluded. Furthermore, patients with less than 15% basophil activation on stimulation with anti-IgE (positive control) were also excluded for the validation experiments.

Pollen sensitization was defined by at least one positive (>0.10 kU_A/L) sIgE result to the following recombinant (r) or native (n) allergens: rBet v 1 the major allergen from birch (*Betula verrucosa*), sIgE against rPhl p 1 and rPhl p 5b from timothy grass (*Phleum pratense*)) and/or nArt v 1 of mugwort (*Artemisia vulgaris*). NsLTP sensitization was quantified by at least one positive sIgE result to the following nsLTP components: rAra h 9 from peanut (*Arachis hypogeae*), rCor a 8 from hazelnut (*Corylus avellana*), rMal d 3 from apple (*Malus domesticus*), rJug r 3 from walnut *(Juglans regia)*, rPru p 3 from peach (*Prunus persica*), nArt v 3 from mugwort (*Artemisia vulgaris*) and rPar j 2 from wall pellitory (*Parietaria judaica*). Furthermore, total IgE and sIgE to bromelain, as a marker for sensitization to cross-reactive carbohydrate determinants (CCD), was quantified. IgE quantifications were performed using the FEIA ImmunoCAP technique (Thermo Fisher Scientific, Uppsala Sweden) according to the manufacturer's instructions.

Preparation of the crude cannabis extract

The crude cannabis extract was produced using an extraction method described by Rudeschko et al. (9) for apple extracts and is characterized by the presence of enzyme inhibitors. This extraction method was chosen because it showed to have good allergen activity in vitro, is suitable for in vivo testing and is a relatively simple extraction procedure.

An amount of 50 g cannabis leaves and flower(buds) was mechanically homogenized at -20 °C in 100 ml precooled diacetone alcohol (4-hydroxy-4-methyl-2- pentanon, Aldrich Chemie GmbH) for 10 min. The homogenate was mixed with 150 ml precooled acetone and equilibrated at -20 °C overnight. After removal of the supernatant by waterjet vacuum. The resulting pulverized remnant was resolubilized in 100 ml phosphate-buffered saline (PBS) by stirring for 1h at 4°C and centrifuged for 45 min at 4°C.

After dialyses the supernatants in the presence of 2.0 mM EDTA, 5.0 mM DIECA, 0.5 mM BAHC, and 0.2 mM PMSF the extract was lyophilized and stored at -20 °C. The enzyme inhibitors added prevent reactions between proteins and plant phenols or decrease protease activity. Ascorbic acid is known to be an antioxidative agent. The protein content of the extracts was determined by the method of Bradford (10).

rCan s 3 production

To identify the mature peptide sequence of the lipid transfer protein of cannabis, complementary DNA (cDNA) was synthetized from total RNA of leaves from *Cannabis sativa L ssp sativa cv Kompolti.* The amplification of the LTP gene was performed with a primer mix deducted from published amino acid sequences (11, 12). Sequence information of the primer mix and amplification details are given in reference (13). The expression of the maltose-binding protein (MBP)-rCan s 3 fusion protein was performed in *Escherichia coli* and purification was performed exactly as previously described for Hev b 12, the LTP for *Hevea brasiliensis (14).*

Basophil activation tests

Basophil activation tests (BATs) were performed as described in detail elsewhere(15). Briefly, pre-warmed heparinized blood samples were stimulated with four concentrations (0.01, 0.1, 1, and 10 µg/mL) of rCan s 3 (expressed and purified as previously described (13)) and a crude extract of *Cannabis sativa*. Anti-human IgE served as a positive control (10 µg/mL, BD Biosciences, Erembodegem, Belgium) and stimulation buffer was used to measure spontaneous CD63 expression by quiescent cells. Analysis of basophil activation was performed using side scatter, anti-IgE and CD203c (BD Biosciences, Erembodegem, Belgium) to characterize the basophils. Subsequently, within this gate, the percentage of activated basophils, i.e. those expressing CD203c++CD63+, was measured. Results were expressed as net percentages of CD63+ basophils, calculated by subtraction of the spontaneous expression from the allergen-induced CD63 expression. All participants exhibited 15% or more anti-IgE mediated basophil activation. Figure 2 shows a representative example of the flow-cytometric analyses of a single CA patient.

Cytometric bead array technique to quantify sIgE rCan s 3

A cytometric bead array was developed to detect sIgE to nsLTP allergens of *Cannabis sativa* (rCan s 3) (16). The method was validated and standardized as previously described (17) by using rBet v 1 (Stallergenes) from birch pollen *(Betula verrucosa) expressed in E. coli.*

Conjugation procedure

Functional beads (BD CBA functional beads; BD Biosciences, Franklin Lakes, NJ) were conjugated according to the manufacturer's procedures with rCan s 3, as described by Pomponi et al. (16). Briefly, 75 µL of microbeads were incubated with 1.9 µL of 1 mol/L dithiothreitol for 1 hour in the dark at room temperature, vortexing the tubes every 15 minutes. Afterward, the microbeads were washed with coupling buffer (BD Biosciences) and centrifuged at 900*g* for 3 minutes, and the supernatant was resuspended in 20 µL of coupling buffer. Ninety microliters of the selected allergens at a concentration of 1 mg/mL were incubated with 2 µL of fresh prepared sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) solution (2 mg/mL; Thermo Scientific, Pierce, Ill) for 1 hour at room temperature in the dark vortexing every 15 minutes. The Bio Spin columns (Bio-Rad, Milan, Italy) were prewashed with coupling buffer, and the protein/sulfo-SMCC mix was transferred to the column, followed by spinning at 1000*g* for 2 minutes. The modified proteins in the eluate were transferred to functional microbeads and incubated for 1 hour at room temperature and protected from light. The tubes were vortexed every 15 minutes. Afterward, 2 µL of N-ethylmaleimide (2 mg/mL in dimethyl sulfoxide, Thermo Scientific) was added for 15 minutes, with vortexing every 5 minutes. After incubation, 1 mL of storage buffer was added, and the tubes were spun for 3 minutes at 900*g*. Washing with storage buffer was performed 3 times before the microbead pellet was resuspended in 500 µL of storage buffer at a concentration of 6×10^6 microbeads/mL and stored at 2°C to 8°C and protected from light before use.

Testing procedure

One microliter of each allergen-conjugated microbead was used for each test. The microbeads were washed with 1 mL of PBS with 0.05% Tween 20 and 0.05% NaN_3(PBS-T) and centrifuged for 5 minutes at 500*g*. After removing the supernatant, the microbeads were resuspended in 50 µL of PBS-T with 1% BSA (PBS-T–BSA).

Fifty microliters of diluted serum samples (1:5 in PBS-T–BSA) was added to 50 µL of the mixed capture beads and incubated for 1 hour at room temperature in the dark. Tubes were then washed with PBS-T and resuspended in 100 µL of PBS-T–BSA after removing the supernatant. Then 50 µL of diluted phycoerythrin-conjugated anti-human IgE (1:100 in PBS 0.05% NaN_3; BioLegend, San Diego, Calif) was added and incubated for 1 hour at room temperature in the dark. The samples were washed with 1 mL of PBS-T, centrifuged for 5 minutes at 200*g*, and resuspended in 200 µL of PBS-T. Afterward, the samples were measured on a FACSCanto II Flow Cytometer (BD Biosciences), and results were expressed as MFI.

Validation

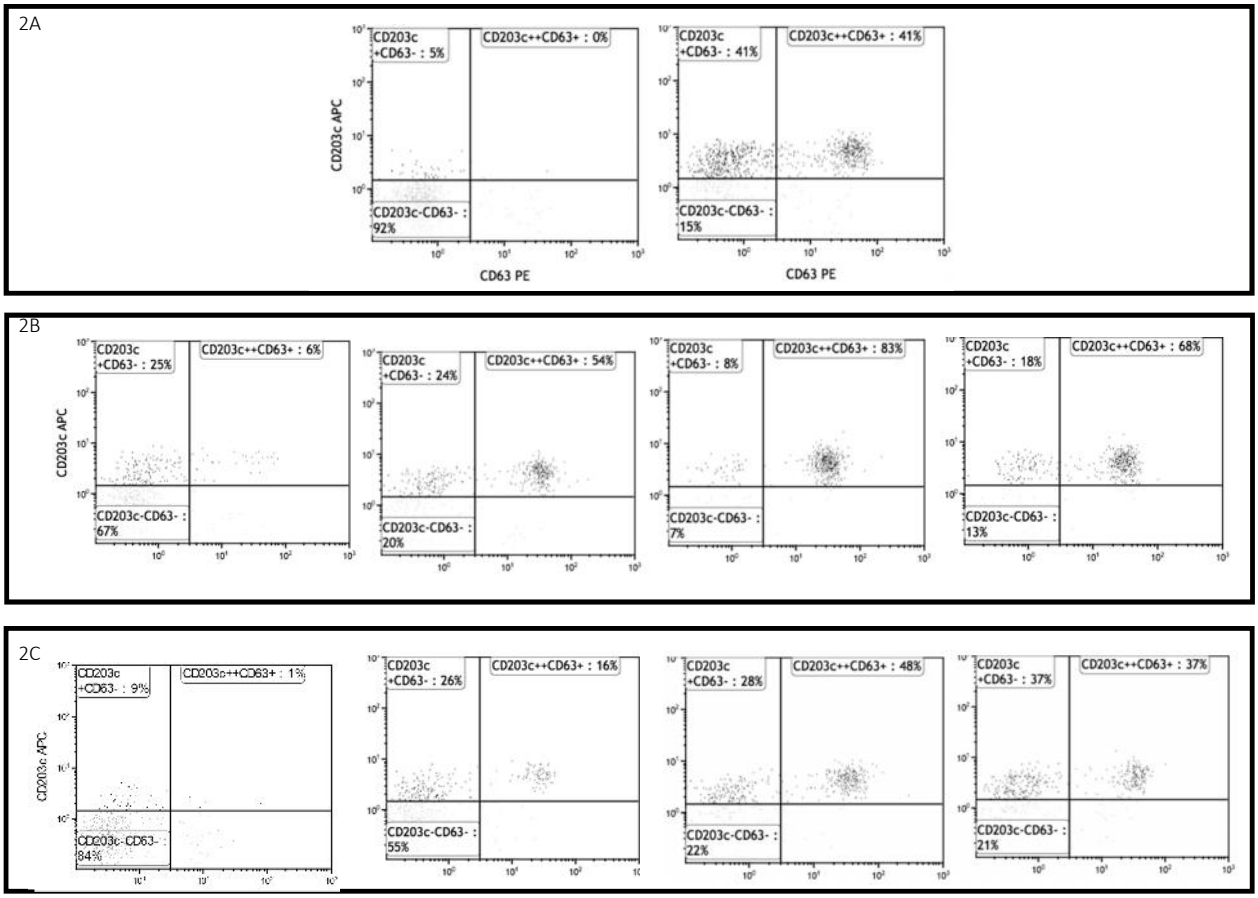
Standardization of the flow cytometric immunobead array was performed with rBet v 1–positive samples compared with the ImmunoCAP technique. At least 5 samples with known rBet v 1 IgE concentrations (ImmunoCAP; in kilounits of allergen per liter) were pooled and tested. A 5-parameter logistic nonlinear standard curve was constructed to normalize the MFI. Reproducibility was tested, and a coefficient of variation of 10% was found.

Statistical analysis

IBM SPSS version 24.0 (IBM, Chicago, Ill., US) software was used for data analysis. Dose finding experiments were performed using a dose-response curve, receiver operating curves (ROC) and comparison of the areas under the curve (AUC) performed using the method described by Hanley et al (18).

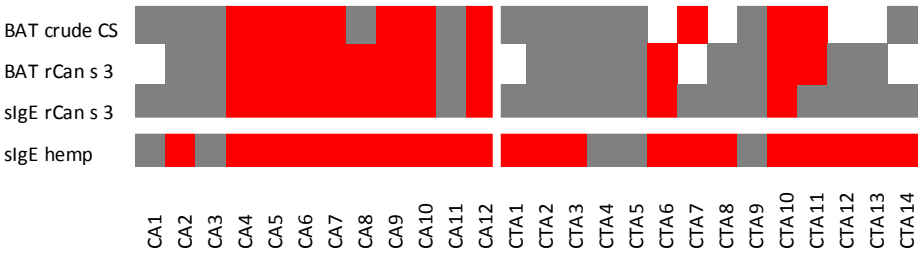
Figures and Tables

Figure 2



Percentage (%) of basophil activation as measured by CD63 expression (x-axis) and CD203c upregulation (y-axis) in a single patient. The first two plots (2A) show the stimulation with anti-IgE and stimulation buffer as negative and positive control respectively. The second panel (2B) shows the dose response experiments with 0.01-0.1-1-10 µg/mL crude cannabis extract whereas the third panel (2C) shows 0.01-0.1-1-10 µg/mL rCan s 3.

FIGURE 3



red= positive test results, grey= negative test results. CA= cannabis allergic individuals, CTA= cannabis tolerant individuals with a pollen and nsLTP sensitization.

TABLE 2

PATIENTS	SYMPTOMS ON EXPOSURE TO CANNABIS	EXPOSURE
CA1	U, D, RC	A,P
CA2	U, C, AE, W	A,I
CA3	U, D, AE	A
CA4	U, AE, D, RC, V*	A
CA5	U, D, RC	A, P
CA6	U, RC, HT	A
CA7	U, D, AE, W	A
CA8	AE, D, N/V	A
CA9	U, RC, N, V	A
CA10	AE, D, RC	A, P
CA11	AE, D, RC	A
CA12	U, D, RC	A

U=urticaria, D=dyspnoe, W=wheezing, C=cough, RC=rhinoconjunctivitis, AE=angioedema, V=vertigo, HT=hypotension N/V=nausea and vomitus. A=active smoking and/or cutaneous contact, P=passive smoking, I=ingestion (hemp seed or cannabis oil)*

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TABLE 3

Patient group		Cannabis allergic (CA=12)	Cannabis Tolerant Atopic (CTA=14)	Healthy Control (HC=15)
Clinical characteristics				
Age (years) Median (Q25-Q75)		29.0 (26.6-34.4)	30.4 (23.2-43.0)	33.9 (28.3-44.3)
Sex ratio *(male: female)*		4:8	9:5	11:4
Atopic dermatitis *with use of topical corticosteroids*		4/12	6/14	0/15
Severity of plant-food allergies*	No food allergy	2/12	2/14	15/15
	Oral allergy syndrome	3/12	8/14	-
	Systemic reactions	7/12	4/14	-
IgE measurements				
Total IgE (kU_A/L) Median (Q25-Q75)		213 (108-368)	458 (123-1220)	23 (8-51)
Bromelain *(>0.10 kU_A/L)*	*positive results*	6/12	4/14	0/15
	mean (range)	0.68 (0-4.69)	0.1 (0-0.47)	
Pollen sensitization (>0.10 kU_A/L)				
Bet v 1 (birch)	*positive results*	8/12	10/14	0/15
	mean (range)	17.97 (0-100)	23.24 (0-82.4)	-
Betv 2 (birch)	*positive results*	9/12	3/14	0/15
	mean (range)	0.29 (0-2.94)	1.73 (0-24)	-
Phl p 1 (timothy grass)	*positive results*	8/12	10/14	0/15
	mean (range)	7.07 (0-30.4)	9.91 (0-39.1)	-
Phl p 5b (timothy grass)	*positive results*	7/12	5/14	0/15
	mean (range)	4.25 (0-18.1)	7.52 (0-78.8)	-
Art v 1 (mugwort)	*positive results*	2/12	2/14	0/15
	mean (range)	0.05 (0-0.46)	0.26 (0-3.45)	-
nsLTP sensitization (>0.10 kU_A/L)				
Pru p 3 (peach)	*positive results*	11/12	13/14	0/15
	mean (range)	11.21 (0-83.0)	2.69 (0-15)	-
Mal d 3 (apple)	*positive results*	11/12	12/14	0/15
	mean (range)	10.71 (0-80.9)	2.02 (0-8.21)	-
Jug r 3 (walnut)	*positive results*	11/12	12/14	0/15
	mean (range)	9.09 (0-68.8)	2.37 (0-12.6)	-
Tri a 14 (wheat)	*positive results*	8/12	5/14	0/15
	mean (range)	2.14 (0-16.3)	0.31 (0-3.12)	-
Ara h 9 (peanut)	*positive results*	9/12	8/14	0/15
	mean (range)	5.1 (0-39.9)	1.09(0-11.9)	-
Cor a 8 (hazelnut)	*positive results*	10/12	8/14	0/15
	mean (range)	3.4 (0-22.5)	1.1 (0-16.8)	-
Par j 2 (parietaria)	*positive results*	3/12	6/14	0/15
	mean (range)	0.87 (0-9.79)	0.83 (0-5)	-
Art v 3 (mugwort)	*positive results*	9/12	3/14	0/15
	mean (range)	0.82 (0-3.10)	0.46(0-6.18)	-

134 **Plant-food allergy includes peach, apple, citrus, kiwi, banana, hazelnut, peanut, walnut and wheat.*

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