

## Evaluation of Skin Reactivity: The concept of Histamine Equivalent Allergen Threshold Concentration ( $C_{ha}$ )

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### Abstract

**Background:** Skin prick testing is the most common diagnostic tool used by allergists. There are limited, international rules on interpreting and reporting skin test results in a meaningful way.

**Aim:** This communication describes methods to express the results of skin prick tests in a meaningful way. It is recommended to use allergen extracts with defined composition, potency and stability, to keep the precision of SPT within acceptable limits by using duplicate tests, and to regularly calculate the c.v. If duplicate tests cannot be performed for practical and or psychological reasons, e.g. in small children, then it is proposed that regular proficiency tests are performed and reported. The method of estimating the allergen threshold concentration, histamine equivalent allergen concentration  $C_{ha}$ , is described

**Conclusion:** By adjusting the allergen wheal response to that of histamine,  $C_{ha}$ , differences in techniques between personnel and centres can be minimized and changes in skin reactivity can be calculated as a threshold concentration. To document the skin reactivity and changes over time it is even proposed to report the mean histamine wheal response of groups and of testing personnel at all-time points of therapeutic trials and in practice over time.

**Keywords:** Skin prick test; Allergen; Histamine; Histamine equivalent concentration; Threshold concentration; Proficiency testing; Cut-off; Precision; Technique

**Abbreviations** A: Area of the allergen or histamine wheal; AAAAI: American Academy of Allergy, Asthma and Immunology; ACAAI: American College of Allergy, Asthma and Immunology; C: Concentration;  $C_{ha}$ : Histamine equivalent allergen concentration; CPT: Conjunctival Provocation Test; CV: Coefficient of Variation; D: Diameter; DBPCFC: Double-blind Placebo Controlled Food Challenge; EAACI: European Academy of Allergy and Clinical Immunology; SD: Standard Deviation; SPT: Skin Prick/Puncture Test; s-IgE: Allergen IgE Antibodies in Serum

### Introduction

The skin prick/puncture test (SPT) is the most common diagnostic tool within allergology. There are many examples of practice parameters or position papers such as "Allergy diagnostic testing: an updated practice parameter" [1], the "EAACI position paper on Allergen standardization and skin tests" [2], "The skin prick test-European standards" [3], and the "Global Atlas of Allergy" [4]. However, despite these statements, most publications using SPT for diagnosis and/or evaluation of changes in skin sensitization do not contain relevant information on the test procedure and does not use meaningful methods for evaluation of results. It is therefore necessary to discuss how to evaluate those results scientifically and clinically.

On the basis of recent publications this brief review aims to discuss the current practices for interpretation of SPT results in clinical trials and daily practice.

### Basics on the skin prick test method

To obtain useful information from skin prick test results, allergens should be standardized, devices with known background and cut-off should be used, and tests be performed according to accepted methods.

Skin prick test results have mainly been given in mm mean diameter or in some scientific papers by wheal area. In 1973, Aas et al. proposed to relate the allergen wheal response to that of histamine [5], with the rationale: When the skin test technique produces small allergen wheals, the histamine wheal is also smaller than usually seen. Forty two years later, Dreborg showed the results obtained by testers with different technique were equalized by determining the histamine equivalent allergen concentration [6],  $C_{ha}$ , using parallel line bioassay and a simple method using one concentration of allergen and one of histamine, based on the allergen dose response relationship [7]. This review will discuss the use of  $C_{ha}$  in clinical practice and scientific work.

### Registration of skin response

After 15 minutes (10-20 minutes) the histamine and allergen wheal should be encircled and then either

Measure the longest diameter ( $d_1$ ) and the midpoint orthogonal diameter ( $d_2$ ), then calculating the mean diameter (D)

$$((d_1+d_2)/2=D) (1)$$

or Calculate the area by planimetry or digitizer. Poulsen et al. developed a simple scanning program to estimate the wheal area [8,9]. This program is no longer in use but there are modern programs for estimation of cell area, e.g. cell sens software [10].

The wheal should be surrounded by a flare [1].

The results should be registered on a record sheet (and in the computer program). Drawings and measurements should be preserved for possible follow up and control.

One report suggests the longest diameter correlates better than the mean diameter with the area of the wheal [11]. However, this correlation does not prove the longest diameter is a better measure than the mean diameter as a measure of skin sensitivity. The question is if there is a better correlation between the patient's shock organ sensitivity and different expressions of skin sensitivity. Such data are not available for the longest diameter. However, changes in conjunctival sensitivity correlate well with changes of wheal area and mean wheal diameter [12].

### There are other methods that have been used for scientific investigations, for example

- estimating the blood flow within and around the wheal area [13],
- estimating electrical impedance [14],
- using thermography [15],
- using digital photography [16],
- and carrying out 3-D scanning [17].

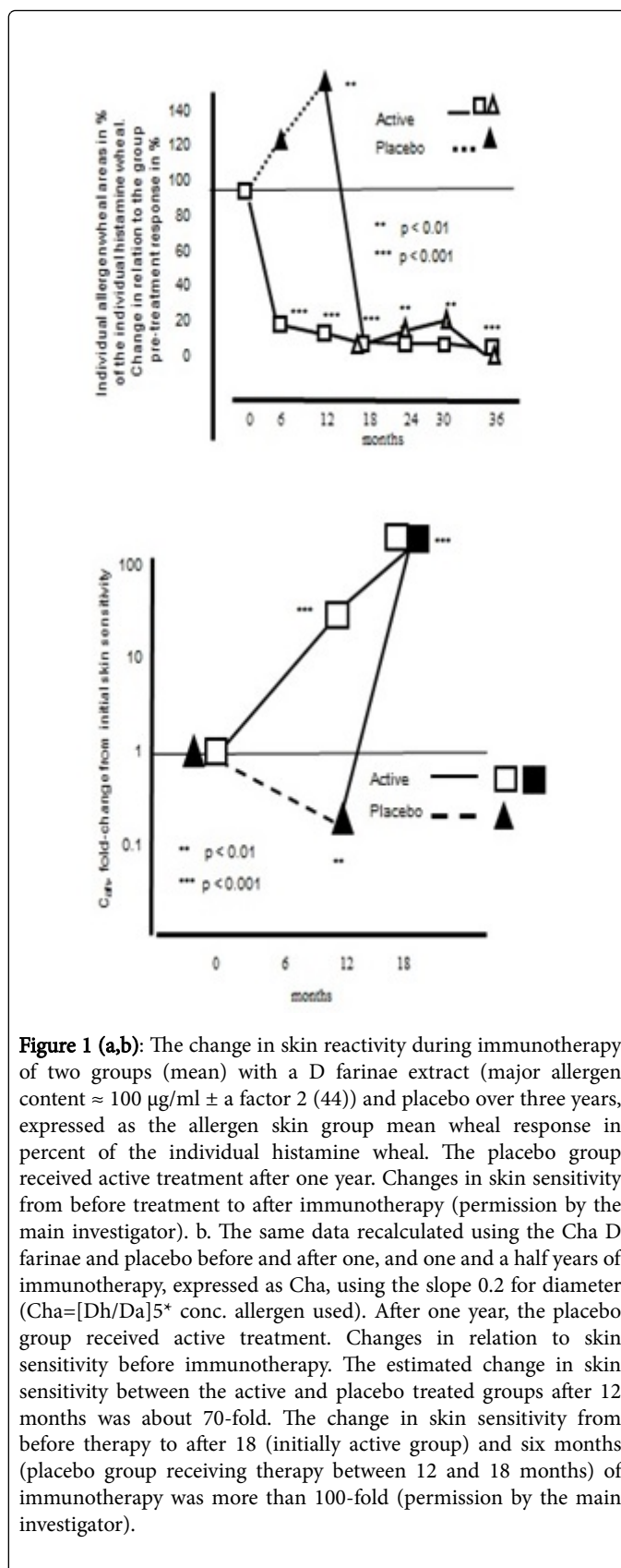
### Evaluation of skin response

- Expression of test results
- In principle, there are five possibilities:
- Using the mean (D) of the longest ( $d_1$ ) and the midpoint orthogonal ( $d_2$ ) diameters,
- Using the area.
- Estimating the allergen mean wheal diameter (wheal area) in relation to that of histamine, using a +++ system [18].

The first three approaches are well known. However, they do not express changes in relevant terms [7]. The third method relates allergen wheal response to that of histamine. However, it is a non-precise, semi-quantitative method not reporting allergen threshold concentrations [18]. Using the method proposed by Durham [19] of using the allergen concentration eliciting a wheal with 6 mm diameter as a threshold concentration. The fourth method does not correct for differences in SPT technique [6] or for changes in histamine sensitivity due to changes in allergen sensitivity over time or due to therapy [12].

### Calculating the allergen wheal size in percent of the histamine wheal size

When using the fifth method allergen sensitivity is related to the histamine reactivity, but it does not give any information on the sensitivity as expressed in allergen concentration and such data cannot be used to determine the degree of change in allergen threshold concentration during e.g. immunotherapy. The changes in a double blind placebo controlled study are shown in Figure 1a.



**Figure 1 (a,b):** The change in skin reactivity during immunotherapy of two groups (mean) with a *D. farinae* extract (major allergen content  $\approx 100 \mu\text{g/ml} \pm$  a factor 2 (44)) and placebo over three years, expressed as the allergen skin group mean wheal response in percent of the individual histamine wheal. The placebo group received active treatment after one year. Changes in skin sensitivity from before treatment to after immunotherapy (permission by the main investigator). b. The same data recalculated using the Cha *D. farinae* and placebo before and after one, and one and a half years of immunotherapy, expressed as Cha, using the slope 0.2 for diameter ( $\text{Cha} = [\text{Dh}/\text{Da}]^{0.2}$  conc. allergen used). After one year, the placebo group received active treatment. Changes in relation to skin sensitivity before immunotherapy. The estimated change in skin sensitivity between the active and placebo treated groups after 12 months was about 70-fold. The change in skin sensitivity from before therapy to after 18 (initially active group) and six months (placebo group receiving therapy between 12 and 18 months) of immunotherapy was more than 100-fold (permission by the main investigator).

### Calculating the histamine equivalent threshold concentration, $C_{ha}$

Finally, the sixth method has the advantage of expressing the result in relation to histamine (6) and is also related to the skin sensitivity (histamine), expressing the allergen sensitivity as the histamine equivalent allergen concentration,  $C_{ha}$  [7].

The same data as in Figure 1a are shown in Figure 1b, showing the  $C_{ha}$  before and after 12 and 18 months of immunotherapy. It demonstrates the about 100-fold difference in change in skin sensitivity between active and placebo treatment from before to after 12 months of immunotherapy and the more than 100-fold reduced skin sensitivity in both groups after 18 and 6 months of immunotherapy, respectively.

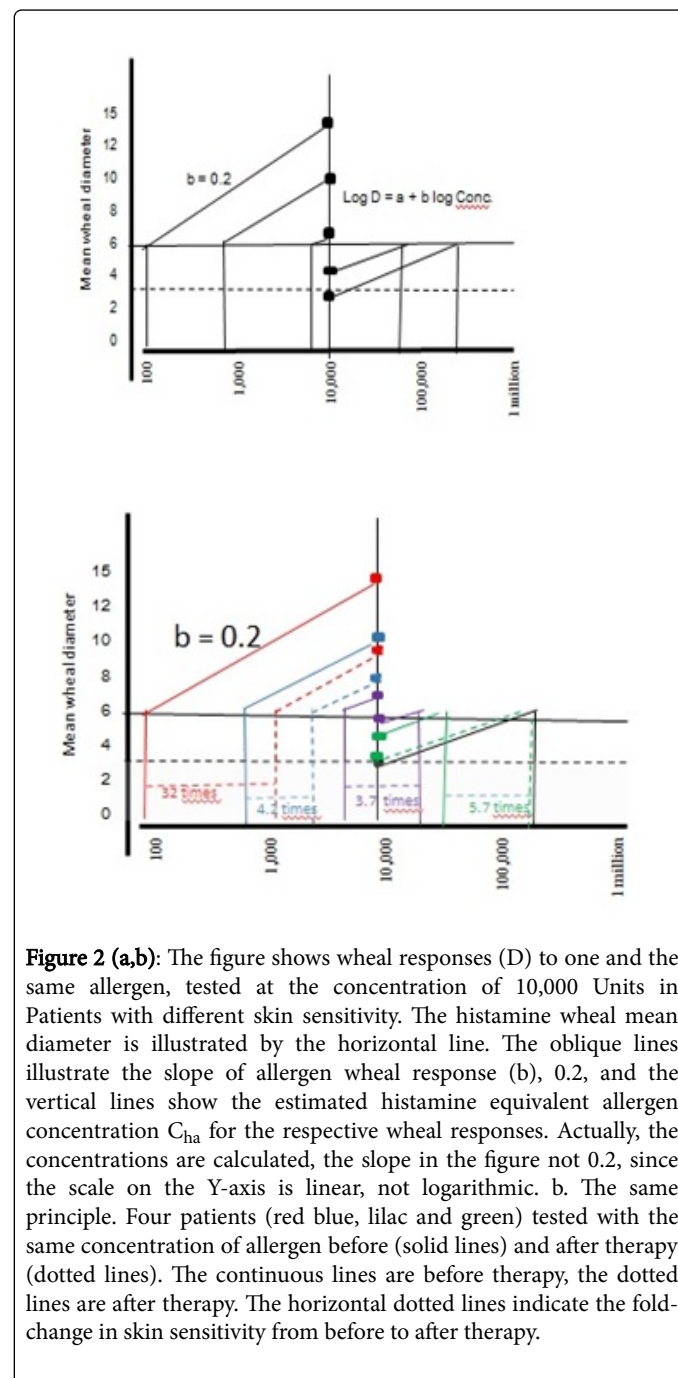
The mean allergen wheal diameter or the allergen wheal area is used in routine and in most published trials. This is simple, but does not give any information about the sensitivity of the patient that is comparable to *in vivo* threshold concentrations ( $PC_{20}$ ,  $PD_{20}$ , CPT/NPT threshold concentrations or the double blind, placebo-controlled food allergen challenge, DBPCFC, threshold concentrations) and *in vitro* tests with a documented cutoff. Furthermore, it does not enable calculation of changes in skin sensitivity to allergens, with or without therapy, as expressed in changed threshold concentration, over time or between groups in cross-sectional studies.

After the introduction of SPT, European manufacturers started delivering extracts in one concentration and, even in the US, it became common to use one dilution of the stock solution for SPT. This method does not deliver an allergen concentration but delivers a diameter (or area).

Prior to the widespread use of SPT, intra-dermal skin testing was used for end-point titration. Then the endpoint, a concentration of allergen, was used as a measure of skin sensitivity and was often used to determine the starting dose for immunotherapy. The end point concentration as determined by SPT correlates with the threshold concentration using the gold standard for estimation of the histamine equivalent allergen concentration,  $C_{ha}$ , the parallel line bio-assay [20,21], and with shock organ sensitivity as expressed by CPT [12,22].

Determining the concentration of allergen eliciting a wheal of the same size as that of histamine reduces the difference between testing personnel, centers and test occasions [6]. Using the slope (b) of the allergen dose response relationship [23], the allergen response can be expressed as a threshold concentration ( $C_{ha}$ ) [7], as illustrated in Figure 2a. The mean slope (b) of the allergen dose-response relationship ( $\text{Log } D$  (mean wheal diameter)=a (intercept with the Y-axis)+b (the slope)  $\log$  (concentration of allergen)) was independently calculated by Dreborg et al. [23-26], and Björkstén et al. [27] (non-published, observation data mentioned in [27]) and was found to be 0.2. The group of Dreborg used parallel line bio-assay [21,22] in several publications for determination of the biological activity of allergen extracts. That material delivered information on the median slope (b) of the allergen dose response relationship. Initial preliminary studies were carried out using histamine dihydrochloride 1 mg/ml [24-26] and then a final study used histamine dihydrochloride 10 mg/ml as standard [23]. In total more than 700 adults (15-50 years) were tested with in-house reference standards (IHR) of two grass species, birch, alder and hazel, mugwort, two species of *Parietaria*, two other weeds (English Plantain and Goose-foot), two pets (cat and dog), two molds (*Alternaria alternata* and *Cladosporium herbarum*), two mites (*Dermatophagoides pteronyssinus* and *D. farinae*). Each species was

tested in at least 20 patients. The pet, grass, tree and mite extracts were tested in several European regions with similar results, i.e. overlapping c.i.. Björkstén et al. [27] tested 708 adolescents (aged 15-17 years) and 220 adults with 43 allergen extracts from different manufacturers to determine the potency of the extracts in HEP and the differences between suppliers.



**Figure 2 (a,b):** The figure shows wheal responses (D) to one and the same allergen, tested at the concentration of 10,000 Units in Patients with different skin sensitivity. The histamine wheal mean diameter is illustrated by the horizontal line. The oblique lines illustrate the slope of allergen wheal response (b), 0.2, and the vertical lines show the estimated histamine equivalent allergen concentration  $C_{ha}$  for the respective wheal responses. Actually, the concentrations are calculated, the slope in the figure not 0.2, since the scale on the Y-axis is linear, not logarithmic. b. The same principle. Four patients (red blue, lilac and green) tested with the same concentration of allergen before (solid lines) and after therapy (dotted lines). The continuous lines are before therapy, the dotted lines are after therapy. The horizontal dotted lines indicate the fold-change in skin sensitivity from before to after therapy.

The histamine equivalent allergen concentration ( $C_{ha}$ ) can be calculated based on the formula [2] derived from the allergen dose response relationship ( $\text{Log } D = a + b \cdot \log C$ ) [7]:

$$C_{ha} = [Dh/Da]^{1/b} \cdot \text{conc. allergen used, (2)}$$

$C_{ha} = [(Dh/Da)]^{1/0.2} \times \text{conc. allergen used}$  or  $C_{ha} = [Dh/Da]^{5 \times \text{conc. allergen used}}$  [7,27]. Formula [3] can then be used to determine the difference in skin sensitivity between two time points or trials.  $C_{ha}$  time one and  $C_{ha}$  time two [3,12]. This describes the differences in concentration, e.g. from before to after therapy or between samples of patients. However, the difference can also be expressed as a ratio providing the fold-increase or decrease in skin sensitivity.  $C_{ha}$  time two/ $C_{ha}$  time one [4,12]

These formulas are easy to introduce in an Excel spreadsheet. The step by step calculation of  $C_{ha}$  is illustrated in Figure e1 in the on line repository. The  $C_{ha}$  is much more useful than the wheal diameter or area and can be used for estimation of changes in skin sensitivity during therapy, e.g. with antihistamines or by immunotherapy [12]. However, when using the results of skin prick tests for this purpose, the technique must be optimal with a c.v. less than 20% (or optimally <10%) using the wheal diameter. Small changes in wheal mean diameter, D, mean large changes in skin sensitivity. A high sensitivity is expressed by lower  $C_{ha}$  values, i.e. larger wheals indicate lower  $C_{ha}$  and smaller allergen wheals correspond to higher  $C_{ha}$ , Table 1.

Table 2 illustrates the relation between changes in allergen wheal diameter, D, and the fold change in skin sensitivity to the tested allergen. It should be noted that small changes in D at low response levels correspond to major changes in skin sensitivity. Changes including wheals less than 3 mm in D are presented in brackets due to the currently generally accepted cut-off at 3 mm in D. However, 2 mm in D is included to illustrate that a change from 3 to 2 mm in D corresponds to a 7.6-fold change in skin sensitivity, i.e. to about 13% of the pretreatment/earlier skin sensitivity. That should be compared to the decrease in symptom scores during antihistamine or cortisone

therapy, mostly to about 70% of pretreatment symptoms, i.e. less than 2-fold.

mm D	$C_{ha}$ in U
15	102
12	313
10	778
9	1 317
7.5	3 277
6.5	6 702
6	10 000
5	24 883
4.25	56 080
3	320 000
2	2 430 000

**Table 1:** An illustration of what small changes in wheal mean diameter, D, means in terms of changed skin sensitivity. The  $C_{ha}$  is calculated with a histamine wheal mean diameter of 6 mm, using an allergen extract labelled 10,000 U. Thus, for a 6 mm allergen wheal,  $D=C_{ha}$  10,000 Units. Like other threshold concentrations, a high sensitivity is expressed by lower values, i.e. larger wheals indicate lower  $C_{ha}$  and smaller allergen wheals correspond to larger allergen wheals.

Difference mm	Fold-change	Difference mm	Fold-change	Difference mm	Fold-change
15-12	3.1	10-7.5	4.2	5-4.25	2.3
15-9	12.9	10-6.5	8.6	5-3	12.9
15-7.5	32.0	10-5	32.0	(5-2)	(97.7)
15-6	97.7	10-4.25	72.1	4.25-3	5.7
12-10	8.6	6.5-5	3.7	(4.25-2)	(43.3)
12-7.5	10.5	6.5-4.2	8.4	(3-2)	(7.6)
12-6	32.0	6.5-3	47.7		
12-5	79.6	(6.5-2)	(362.6)		

**Table 2:** The table illustrates the relationship between changes in allergen wheal diameter, D, and the fold-value of the change in skin sensitivity to the tested allergen. It should be noted that small changes in D at low response levels corresponds to major changes in skin sensitivity. Changes including wheals less than 3 mm in D are shown in brackets due to the current generally accepted cut-off at 3 mm. It should be noted that a change from 3 to 2 mm allergen wheal D corresponds to a change in skin sensitivity of around 7-fold, i.e. to about 13% of the pre-treatment sensitivity. In comparison the decrease in symptoms during antihistamine or cortisone therapy is mostly found to be about 70%, i.e. less than a 2-fold change in symptom scores.

In Figure 1b the mean allergen wheal sizes in % of the mean histamine wheal diameter (Figure 1a) have been recalculated into  $C_{ha}$  before and after one year of immunotherapy [12,28]. Another theoretical example of results using  $C_{ha}$  before and after immunotherapy is shown in Table 3. One allergen is used for immunotherapy, the reactivity to the other is just observed, such as by Dreborg et al. [29]. It has been shown that the histamine reaction is

reduced during immunotherapy [12,30]. The calculated change in sensitivity to allergens is influenced by the change in histamine reactivity as illustrated in the table. However, the difference in changes between active and placebo does not change. This is an important observation. The difference in changes in relation to non-treatment or placebo is the crucial parameter when evaluating any type of anti-allergic therapy.

Group	Before immunotherapy			After immunotherapy			Fold-change group	within	Fold-difference change	in
	Dh	Da	$C_{ha}$	Dh	Da	$C_{ha}$				
Histamine wheal unchanged during immunotherapy										
Active allergen	6	6	10	6	3	368	36.8		36.8	
Placebo allergen	6	6	10	6	6	10	1			
Histamine wheal reduced by 1 mm during immunotherapy										
Active allergen	6	6	10	5	3	142	14.2		36.8	
Placebo allergen	6	6	10	5	6	4	0.4			
Histamine wheal reduced by 2 mm during immunotherapy										
Active allergen	6	6	10	4	3	45	4.5		36.8	
Placebo allergen	6	6	10	4	6	1	0.1			

**Table 3:** One allergen is used for immunotherapy, the reactivity to the other is just observed. Three examples are shown in the table. The response to histamine is the same or is reduced by 1 and 2 mm diameter during immunotherapy. The calculated changes in sensitivity to the respective allergens are influenced by the change in histamine reactivity, as illustrated by the three cases. However, the differences in change are the same.

### Factors of importance for meaningful determination of $C_{ha}$

#### Cutoff

The question of when a test is positive or negative has been subject to lively discussion as long as skin testing has been part of allergy diagnosis. In studies, as well as in clinical practice, defining positive and negative results is important.

Actually, for decades there was no agreement on how to define a positive SPT. In 1987, Dreborg et al proposed  $\geq 7 \text{ mm}^2$  ( $\approx \geq 3 \text{ mm D}$ ) to be the cut-off that was adopted by the Nordic Guidelines [31] and the EAACI position paper [2]. However, there was no documentation

of that limit at that time. One factor influencing the proposal was the higher c.v. at low response levels [23,26]. There must be a clear definition of the background using the device [1], diluent and technique used in the office/study. The cut-off should be the upper limit of the background, i.e. the background mean +3.3 standard deviations (s.d.) [32]. The background is determined by testing a number of patients with a number of tests with the negative solution. Later, Nelson et al. [33,34] reported the background of a number of devices used in the US, using 80 tests with a negative control solution and calculating the background [33,34], and thereby the cut-off, in agreement with [32] (Table 4). For devices unique to Europe and other areas, data are missing. For those, the cut-offs should be defined.

Device 1	0.99 Quintile of reactions at the negative control sites, mm	Device 2	0.99 Quintile of reactions at the negative control sites, mm
Devices for which a 3-mm wheal would be significant		Devices for which a more than 3-mm wheal should be used as significant.	
Quintest (HS) puncture	0	DuoTip (Lincoln) twist	3.5
Smallpox needle (HS) prick	0	Bifurcated needle (ALO) prick	4
DuoTop (Lincoln) prick	1.5	MultiTest (Lincoln) puncture	4
Lancet (HS)	2	Bifurcated needle (ALO) puncture	4.5
Lancet (ALK)*	3	Quick Test (Pantrex)	4
DermaPICK II	0	Greer Track (Greer)	3.5

HS, Hollister Steer; Greer, Greer Laboratories; ALO, Allergy Labs of Ohio; Lincoln, Lincoln Diagnostics; ALK, ALK.\*Not the ALK lancet used in Europe.

**Table 4:** The size of wheals that are larger than 99% of the wheals with saline, using the same device on the subject's back by the same operator (n=80) [1]. These data are US data and are not applicable to European devices.

## Precision

The reliability of the results of diagnostic tests is depending on the precision of the estimate. This is true for SPT and is of major importance when evaluating SPT results for screening, diagnosis as well as using  $C_{ha}$  as a measure of the response.

*In vitro* tests should always have a documented precision (c.v.). The c.v. of in vitro tests is most often low, less than 10%. There are few reports on the precision of SPT. Aas [35] reported the precision (C.V.) of SPT based on the mean wheal diameter, using the methods of Pepys [36,37], Brown [38] and a multi-test device. The C.V. varied from 8% using the Pepys' method [36], with a short beveled needle, to 30% with a multi-test device using the mean wheal D (Table 5). In major European centers [23], the C.V. varied from 15% up to 145% for allergens and from 12% to 65% for histamine, as calculated on wheal areas using the Osterballe needle with 1 mm point and shoulders preventing further penetration [39]. The c.v. calculated on the diameter is half that of the c.v. calculated on the area (area of a circle  $\pi r^2$ , the index 2 makes the difference). In that report, quadruplicate tests with each of three concentrations of allergen (about 9000 tests) and histamine dihydrochloride 1 and 10 mg/ml was used (about 5000 tests). However, duplicate tests are sufficient for calculation of the c.v. Examples are shown in Figure 3 (a,b) and Table 6.

Device	n	D	s.d.	c.v.	Range
Short beveled needle	80	6.1	0.51	8	05-Jul
Morrow Brown needle	80	5.7	0.55	10	4.5-7
Multi-test (brand not defined)	80	4.5	1.35	30	02-Jul

**Table 5:** Tests performed according to Pepys using a short beveled needle, with the Morrow-Brown needle and with a multi-test device, testing the same 80 patients with histamine dihydrochloride 1 mg/ml, according to Aas. This assistant should obviously use the method of Pepys, causing the largest wheals, the lowest range of histamine weal sizes and the lowest c.v. Dis the mean diameter, i.e. mean of the longest and the midpoint orthogonal diameters; s.d. is the standard deviation; c.v. is the coefficient of variation.

## Documentation of the precision of the skin prick test method

In principle, there are (at least) two possibilities: To perform duplicate tests with histamine and all allergens, or to use proficiency testing [1,40] (and in the online repository). In small children single tests are acceptable, provided proficiency tests are performed at intervals.

## Duplicate tests

In most cases, it is easy to perform duplicate tests instead of single tests. The extra time spent is limited. The extra material needed is negligible too, since the same device/needle/lancet can be used for the second test with the same allergen in the same patient. When performing Prick-Prick tests a new device should be used for each patient, allergen, but can be used for two pricks with the same allergen in the same patient.

The precision is given by the coefficient of variation, c.v., which is derived from the formula:

$$Sd \times 100 / \text{mean} = \text{c.v. percent (5)}$$

Test	Tester 1			Tester 2		
	d <sub>1</sub>	d <sub>2</sub>	d <sub>1</sub> +d <sub>2</sub> /2	d <sub>1</sub>	d <sub>2</sub>	d <sub>1</sub> +d <sub>2</sub> /2
			D			D
1	8.5	6.5	7.5	5	4	4.5
2	9	7	8	5	3	4
3	10.5	6	8.25	5	5	5
4	9	6	7.5	5	3.5	4.25
5	10	6	8	5	5	5
6	10	6	8	4	3.5	3.75
7	9	6.5	7.75	5	5	5
8	7	6	6.5	6	4	5
9	8.5	6	7.25	6	5	5.5
10	8	6	7	5.5	4	4.75
Median	9	6	7.6	5	4	4.9
Mean	9	6.2	7.6 p<0.0000 15.2	0	4.2	4.7
s.d.	1	0.3	0.5	0.6	0.8	0.5
c.v. %	11.6	5.6	7.1	11.3	17.9	11.6
Range	7-10.5	6.0 - 7.0	6.5 - 8.25	6.0 - 4.0	3.0 - 5.0	3.75 - 5.5
Significance of difference between means						
Histamine conc. for the same result		10 mg/ml			171 mg/ml	

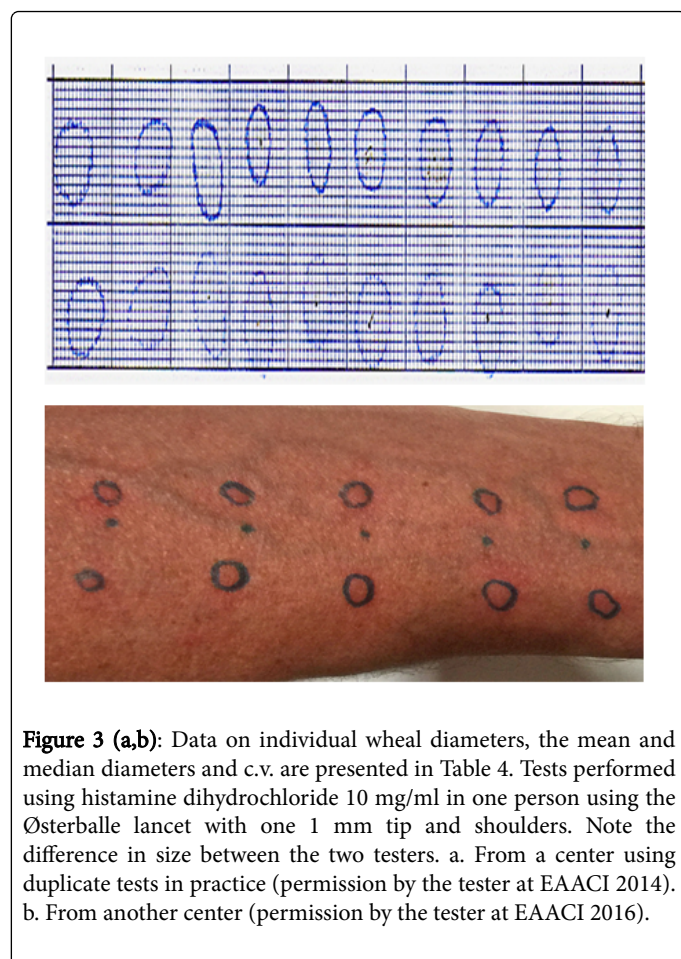
**Table 6:** Prick tests performed on the volar aspect of the forearm by two testers using histamine dihydrochloride 10 mg/ml, Figure 4 (a,b). The measured diameters (d<sub>1</sub> and d<sub>2</sub>) and the mean of these (D) of ten wheals are shown. The table also shows the mean and median of D of the ten tests and the c.v. The mean diameter, D, and c.v. are calculated by formulas in the cells of an Excel sheet in the on-line repository. To the left are the results for a tester with a high pressure on the lancet. To the right the results are shown for a tester applying low pressure on the lancet.

When ocularly comparing two tests, the rule is that at normal response levels, between approximately 4 and 8 mm D, a difference of  $\pm 1$  mm can be accepted. At the same time it should be remembered that the difference in strength of an extract causing a 4 mm wheal D to that causing a wheal with a D of 8 mm is around 32-fold [41]. Thus, if an extract that is labelled 10 U gives a 4 mm wheal D, then an extract labelled 320 U induces a wheal with about 8 mm D in the same patient. Similarly, a change in wheal D from 8 to 4 mm during therapy indicates 32-fold reduced skin sensitivity, i.e. less than 3% of the pre-treatment skin sensitivity, and the difference between 4 and 6 mm, i.e. 5

$\pm 1$  mm wheal is 7.6-fold and  $4 \pm 1$  mm means a 13-fold difference in skin sensitivity.

Suggested Proficiency Testing and Quality Assurance	
<b>Technique For Prick/Puncture Skin Testing</b>	
<ul style="list-style-type: none"> <li>Using desired skin test drive, perform skin testing with positive (histamine 1-10) and negative controls (saline 1-10) in an alternate pattern on a subject's back.</li> <li>Record histamine results at 8 minutes by outlining wheals with a felt tip pen and transferring results with transparent tape to a blank sheet of paper.</li> <li>Record saline results at 15 minutes by outlining wheal and flares with a felt tip pen and transferring tape to a blank sheet of paper.</li> <li>Calculate the mean diameter as <math>(D+d)/2</math>; D=largest diameter and d = orthogonal or perpendicular diameter at the largest width of D</li> </ul>	
<b>Histamine</b>	
<ul style="list-style-type: none"> <li>Calculate the mean diameter of each wheal</li> <li>Calculate the s.d.</li> <li>Determine the coefficient of variation (CV) = s.d. /mean or the c.v. % = s.d. * 100/mean</li> <li>Quality standard should be CV less than 30% saline</li> </ul>	
<b>Saline</b>	
<ul style="list-style-type: none"> <li>All negative controls should be <math>\leq 3</math> mm wheals and flares should be <math>\leq 10</math> mm in Diameter.</li> </ul>	

**Table 6:** According to the AAAAI Practice Parameter 2008, Bernstein et al. [1].



**Figure 3 (a,b):** Data on individual wheal diameters, the mean and median diameters and c.v. are presented in Table 4. Tests performed using histamine dihydrochloride 10 mg/ml in one person using the Østerballe lancet with one 1 mm tip and shoulders. Note the difference in size between the two testers. a. From a center using duplicate tests in practice (permission by the tester at EAACI 2014). b. From another center (permission by the tester at EAACI 2016).

Furthermore, it must be considered that in most diagnostic systems, the c.v. increases at lower response levels close to the cut off concentration. In two reports [23,25], the c.v. was reported at different response levels. In both reports the c.v. of allergen SPT was about 50% at wheals 7-10 mm<sup>2</sup> (3-3.5 mm diameter), at higher levels about 30%

with narrow confidence limits. Thus, the proposed simple control method using the  $\pm 1$  mm is not based on solid scientific data, but should be considered a minimum requirement in practice.

### Proficiency tests

The first aim of proficiency testing [1,40] is to train assistants to achieve high precision, both for clinical trials and for improving the value of SPT in clinical practice. The second aim of proficiency testing is to train all assistants in an office or in offices participating in multicenter clinical trials to obtain the same size of the histamine wheals in the same patients. It is proposed this size should be 7 mm in diameter. The aim is to obtain comparable results from the different nurses or centers. It also makes it possible to note at about 70-fold decreased skin sensitivity using 3 mm as cut off and more than 500-fold decrease using 2 mm mean wheal diameter as cut off. The third aim of proficiency testing is to maintain high precision when using single tests, e.g. in children. Then, the testing personnel must document a consistent technique at intervals. Such a test is illustrated in Figure 3a and 3b and the results are shown in Table 5.

The AAAAI practice parameter [1] relates a proposal for proficiency testing; Table 6. However, there are no detailed proficiency test protocols published. Therefore, the protocol used during workshops on skin testing during recent AAAAI Annual Meetings has been added to this paper [40] (online repository, Figure e3). In addition, simple Excel sheets are added in the on-line repository to show how to record, estimate means, calculate c.v. using inserted formulas and store histamine wheal diameters, Figure 4. When the basic skills have been obtained, the proficiency testing protocol proposes to perform 10 + 10 tests with the histamine reference on the volar aspect of the forearms on each of 4 subjects at monthly intervals. Preferably, the same individuals should be used from time to time, the c.v. and the median histamine wheal size recorded and stored to supervise changes in precision and test technique. The c.v. should be maintained at less than 20% (best <10%) and the mean histamine mean diameter (D) maintained at 7 mm diameter. A drawback with single tests is the risk of negative tests due to low pressure on the lancet, causing false negative tests (false negative sensitization). In the above mentioned study [23] tested with 6 allergen concentrations in quadruplicate tests

per patient (n 24), there was an incidence of accidental negative SPT of about 1/20 tests (non-published data), varying between centers.

### The effect of pressure on the lancet/device

Clinically, it has been obvious that the higher the pressure on the lancet, multi-test etc., the larger the histamine or allergen wheal response. However, the first well designed study on the importance of differences in pressure on the device was published recently by Andersen et al. [42]. They used an equipment delivering an exact, predetermined pressure on the lancet, for the first time proving the association between pressure applied and the size of the skin wheal response. The effect of using different test techniques by different testing personnel and between centers participating in multicenter studies is illustrated in Table 5. Both testers used histamine dihydrochloride 10 mg/ml and the ALK-Abelló variant of the Østerballe needle with 1 mm tip [43]. Using the mean wheal diameters (7.58 and 4.68 mms) and the median slope of histamine dihydrochloride (histamine HCl,  $b=0.17$  [23]), results were obtained by applying the formula:

$$[\text{Dh High pressure}/\text{Dh Low pressure}] \times 5.88 \times 10 \text{ (histamine conc.)},$$

(6)

$$[7.58/4.68]5.88 \times 10 = 1.625.88 \times 10 = 17.06 \times 10 = 171.$$

Thus, it seems the tester applying low pressure on the lancet would have needed to use a histamine solution of 171 mg/ml to obtain the same result as the high pressure tester using histamine dihydrochloride 10 mg/ml, i.e. the tester applying low pressure needed a 17-fold higher histamine (and allergen) concentration to get the same result as that obtained by the tester with high pressure. It also means that the low pressure tester will get negative tests (defined as  $<3$  mm mean D) when testing all patients developing a wheal  $<5.3$  mm D when tested by the high pressure tester (using the slope of the allergen dose response [23,24,27] and applying the cutoff of 3 mm). Thus, the results e.g. in epidemiological studies with testers with the illustrated techniques, testing in two different centers, including patients on the basis of positive SPT to a certain allergen, will include quite different patient samples. The high pressure tester will include many patients with lower sensitivity to the allergen tested than the tester with low pressure. These patients will be found not to be sensitized by the low pressure tester. Therefore, in multicenter studies it is important to report on the mean histamine wheal response in all participating centers and by calculation of the skin sensitivity as  $C_{ha}$  and accepting patients with a given  $C_{ha}$  instead of a defined wheal diameter. Using the  $C_{ha}$ , skin test results are made more comparable between testing personnel and centers.

### Limitations and contributions of the $C_{ha}$ method for estimation of skin reactivity

#### Contributions of estimation of $C_{ha}$ for estimation of skin reactivity:

In scientific work, the histamine equivalent allergen concentration,  $C_{ha}$ , can be calculated by parallel line bioassay that needs testing with at least three ten-fold concentrations of allergen and histamine HCl 10 mg/ml, all four in at least duplicate (8 prick tests) and complicated mathematical procedure. Calculation of  $C_{ha}$  using equation 2) needs only the use of histamine 10 mg/ml and one allergen concentration both tested in at least duplicate (4 tests) using the simple equation 2) that can be inserted in e.g. an Excel sheet automatically delivering the result. The methodology is simple and should be obligatory in scientific studies and even useful in clinical practice. The  $C_{ha}$  delivers a threshold

concentration making possible estimation of changes of skin sensitivity in terms of change in concentration similar to what is obtained by all other challenge tests

### Limitations

The only limitations are the estimation of  $C_{ha}$  necessitates training to obtain meaningful data the training has been questioned by some assistants and doctors since it is perceived as a threat against their professional skill.

#### Conclusions and suggested recommendations

Based on previous documentation it is recommended:

- Using well standardized extracts/components, with known total allergenic potency with known stability.
- Using a negative control solution for documentation of each person's background.
- Using a positive control for documentation of skin reactivity, technician's skill and for evaluation of skin reactivity.
- Registering and store the wheal (and erythema) size.
- Determining the mean wheal (and erythema) diameters or areas.
- Aiming at 7 mm mean histamine diameter, using 10 mg/ml in defined patients.
- Documenting the precision by duplicates tests and calculated c.v., or if this is not possible, the c.v. obtained by proficiency tests performed at intervals, should be reported.
- Training personnel to obtain the highest possible precision and regularly report their c.v. as mean histamine diameters

### Recent advances described and recommended are summarized below

Methods for estimation of the histamine equivalent allergen concentration,  $C_{ha}$ , i.e. the proposed SPT threshold concentration.

Methods for estimation of changes of  $C_{ha}$  during therapy and over time, allowing for estimation of change in threshold concentration and or fold-change in threshold concentration.

Relevant parts of these recommendations should be applied also to clinical practice.

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