

## SUPPLEMENTARY MATERIAL

**Supplementary Figure 1. Study design.** Basophil activation test was performed to 40 drug-allergic individuals to amoxicillin, clavulanic acid, cefuroxime, ceftriaxone, dipyrone or azithromycin. with a total of 430 measurements Samples were analysed by Eq#1 and Eq#2, in parallel to perform correlation, concordance and level of agreement between instruments. More information can be found under the "Study and experimental conditions" section of this online supporting material.

**Supplementary Figure 2. Gating strategy for basophil activation measurement** based on %CD63, CD203c<sup>high</sup> expression, total CD203c MFI, CD203c  $\Delta$ MFI (MFI from stimulated sample -MFI from non-stimulated sample) and CD203c MFI ratio (MFI from stimulated sample/MFI from non-stimulated sample). Leukocytes were first gated based on forward and side scatter and basophils were gated according to low side scatter versus CCR3 (CD193) expression (between 500-1000 basophils were evaluated). Activated SSC<sup>low</sup>/CD193- gated basophils were identified in a CCR3/CD203c<sup>high</sup> gate or in a CCR3-CD63 gate. Percentage of CD203c<sup>high</sup>, %CD63 or MFI of CD203c+cells were evaluated as comparators of both instruments. Data acquisition was done with Cell Quest Software and raw data files were exported and analyzed with Flow Jo software (Flow Jo, 10.8.1). An analysis template and workflow were set and not changed between samples. Figure shows representative dot plot of the same sample analysed by both instruments, either unstimulated or stimulated with anti-IgE antibody: A) SSC vs FSC, B) basophils gated by SSC<sup>low</sup>/CCR3, C) percentage of CD203c<sup>high</sup> of gated basophils, with CD203c MFI depicted inside the dot plots. D) percentage of CD63 of gated basophils.

**Supplementary Figure 3.** Analyses of correlation between Eq#1 and Eq#2 with (A) %CD63, (B) %CD230c<sup>high</sup> and (C) CD203c  $\Delta$ MFI. Values of the different readout parameters were plotted Y=Eq#2 vs X= Eq#1. Linearity and correlation were calculated.  $r$  = Pearson's correlation. N=430

**Supplementary Figure 4.** Analyses of concordance between Eq#1 and Eq#2 using Bland-Altman plots for (A) %CD63, %CD230c<sup>high</sup> and (B) CD203c MFI (total MFI,  $\Delta$ MFI and MFI ratio). Green lines  $\pm 1.96$  times standard deviation, n= 430 Bias is indicated beside each plot.

**Supplementary Figure 5. Cohen's kappa coefficient (kc) for level of agreement assessment.** Data obtained by Eq#1 and Eq#2 with % and SI of CD63 and CD203c<sup>high</sup> was translated into a positive or negative result, using either 2.5% or 5% thresholds or stimulation index (SI)>2 with respect to the unstimulated sample. K was obtained from the dichotomic variable of each tube (#observations=430) and from the final test result of each sample (#observations = 72). The interpretation of the k considering all the observations allow to calculate the analytical level of agreement. k obtained with the final test results was used to calculate the clinical level of agreement.

**Study and experimental conditions.** From August to October 2022, BAT was performed following a standard operating procedure (SOP) validated by participating in a multicentric External Quality Assurance (EQA) program,[1] (EQA involves testing of certain parameter or analyte by a number of different laboratories with the purpose to periodically assess the quality of the performance of a certain technique). Anonymized clinical samples from patients with confirmed allergy to amoxicillin, clavulanic acid, cefuroxime, ceftriaxone, dipyrone or azithromycin were analyzed, in parallel in two flow cytometers (Supplementary Figure 1) (FACS Calibur model 2012: Comparator Instrument (Eq#1) and FACS Calibur Model 2012: Test Instrument (Eq#2)), totalizing 456 measurements. Briefly, 100ul of heparin whole blood was incubated with or without the culprit drugs, as well as with anti-IgE (positive control for IgE-

dependent activation) and fMLP (positive control for IgE independent activation), or stimulation buffer alone (negative control, or unstimulated control) at previously defined concentrations, for 25 minutes at 37°C in a water bath, in IL-3-containing stimulation buffer with FITC-antiCD63, PE-antiCD203c and APC-antiCCR3 antibodies. Cell activation was cold-stopped, red-blood cells lysed, and samples were analyzed, immediately or after 24 hours at 4°C, first on Eq#1 and the remaining volume was analyzed on Eq#2, to eliminate any bias due to pipetting errors. Prior to sample acquisition, both instruments were calibrated with Beckton Dickinson (BD) calibration beads, following the manufacturer's instructions. A CCR3<sup>+</sup>/CD203c<sup>+</sup> gate corresponding to total basophils was set and around 1000 events were acquired (a minimum of 500 events were accepted). Activated basophils were assessed either by %CD63, %CD203c<sup>high</sup> (representing the percentage of basophils overexpressing CD203c as a result of activation) or total CD203c mean fluorescence intensity (MFI), CD203c ΔMFI (MFI from stimulated sample -MFI from non-stimulated sample) and CD203c MFI ratio (MFI from stimulated sample/MFI from non-stimulated sample). We studied two thresholds (2.5% and 5%), in agreement with the European Academy of Allergy and Clinical Immunology (EACCI) member laboratories. In addition, stimulation index (SI) was calculated for each readout parameter (SI: ratio of the stimulated basophils obtained after incubation with the culprit versus the unstimulated sample). Assessment of the positive population in activated samples was done considering a net activation of 2.5% or 5% as positive. Correlation studies were done on the continuous variables (%CD63, %CD203c<sup>high</sup> or MFI CD203c) and the binary results (Final positive or negative test results based on a 2.5% or 5% threshold or SI>2). Bland-Altman was used to investigate the level of agreement of both methods, Pearson's correlation to evaluate linear correlation between instruments and Cohens kappa coefficient (k) to assess the clinical significance of concordance.

**Risk-based approach overview.** A risk assessment was done following ICH guideline Q9 on quality risk management, using a cause-effect-based empirical evaluation internal procedure. Briefly, the main hazards were proposed and consequences on correlation were tested. The ICH guideline Q9 was applied, since it is a general tool to identify hazards and risks, and also to evaluate the impact on performance that can be applied to a technique, efficacy of a methodology, efficacy of a product and in this case, performance of two flow cytometers.

### ***Risk assessment***

What might go wrong? 2. What is the likelihood it will go wrong? 3. What are the consequences?

#### 1.1 Risk identification

Potential hazard #1: Differences in PMT voltage between instruments can lead to different SSC vs FSC clouds and different % of basophils in the SSC vs CCR3 window. Potential hazard #2: Differences in PMT voltages can affect MFI of activation markers.

#### 1.2 Risk analysis

Science based questions: Does voltages need to match or rather be adjusted to match MFI? Will gates change due to voltage adjustments to match both instruments? If so, would it affect expression results? How can this be measured? How can this be quantified?

#### 1.3 Risk evaluation

A level of acceptance has to be set. If the consequence of the risk is above the accepted levels then PMT voltage might be a risk. Continue like this with hazard #2,3, etc

#### 2.1 Risk control

Is the risk above an acceptable level? What can be done to reduce or eliminate risks? What is the appropriate balance among benefits, risks and resources? Are new risks introduced as a result of the identified risks being controlled?

#### 3.1 Risk reduction/Acceptance

Among all identified risks which gave an effect below acceptance levels? If none, can any of them be reduced to acceptance?

## References

1. Pascal M, Edelman SM, Nopp A, Möbs C, Geilenkeuser WJ, Knol EF, Ebo DG, et al. EAACI task force report: A consensus protocol for the basophil activation test for collaboration and external quality assurance. *Allergy*. 2023 Oct 5. doi: 10.1111/all.15907.