

## Video Article

# Basophil Activation Test for Investigation of IgE-Mediated Mechanisms in Drug Hypersensitivity

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## Abstract

Hypersensitivity reactions against non-steroidal anti-inflammatory drugs (NSAIDs) like propyphenazone (PP) and diclofenac (DF) can manifest as Type I-like allergic reactions<sup>1</sup>. In clinical practice, diagnosis of drug hypersensitivity is mainly performed by patient history, as skin testing is not reliable and oral provocation testing bears life-threatening risks for the patient<sup>2</sup>. Hence, evidence for an underlying IgE-mediated pathomechanism is hard to obtain.

Here, we present an *in vitro* method based on the use of human basophils derived from drug-hypersensitive patients that mimics the allergic effector reaction *in vivo*. As basophils of drug-allergic patients carry IgE molecules specific for the culprit drug, they become activated upon IgE receptor crosslinking and release allergic effector molecules. The activation of basophils can be monitored by the determination of the upregulation of CD63 surface expression using flow cytometry<sup>3</sup>.

In the case of low molecular weight drugs, conjugates are designed to enable IgE receptor crosslinking on basophils. As depicted in **Figure 1**, two representatives of NSAIDs, PP and DF, are covalently bound to human serum albumin (HSA) via a carboxyl group reacting with the primary amino group of lysine residues. DF carries an intrinsic carboxyl group and, thus, can be used directly<sup>4</sup>, whereas a carboxyl group-containing derivative of PP had to be organochemically synthesized prior to the study<sup>1</sup>.

The coupling degree of the low molecular weight compounds on the protein carrier molecule and their spatial distribution is important to guarantee crosslinking of two IgE receptor molecules. The here described protocol applies high performance-size exclusion chromatography (HPSEC) equipped with a sequential refractive index (RI) and ultra violet (UV) detection system for determination of the coupling degree.

As the described methodology may be applied for other drugs, the basophil activation test (BAT) bears the potential to be used for the determination of IgE-mediated mechanisms in drug hypersensitivity. Here, we determine PP hypersensitivity as IgE-mediated and DF hypersensitivity as non-IgE-mediated by BAT.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/3263/>

## Protocol

### 1. Preparation of drug conjugates

1. Dissolve 10 mg DF in 1 ml dH<sub>2</sub>O in a 1.5 ml reaction tube. In the further proceeding use 95 µl of this DF solution. To conjugate PP to HSA dissolve 30.4 mg PP derivative (304 g/mol) in 500 µl 0.3 M sodium hydroxide (NaOH).
2. Add 430.6 µl dH<sub>2</sub>O and 100 µl 0.5 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer pH 6.5 to 95 µl of dissolved DF. Add 33.1 µl dH<sub>2</sub>O and 140 µl of 2 M MES buffer pH 2.9 to the PP sample.
3. Add 57.5 µl of a freshly prepared *N-ethyl-N*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) stock solution dissolved in dH<sub>2</sub>O with a concentration of 100 mg/ml to the DF sample, or 10 µl EDC solution to the PP sample. Vortex the samples for 1 minute.
4. Add 16.9 µl of HSA solution with a concentration of 118 mg/ml to each sample.
5. Incubate the samples for 2 hours at room temperature while shaking at 300 rounds/min.
6. Dialyze the DF and PP samples 3 times against 2 liters of phosphate-buffered saline (PBS) pH 7.4 for several hours each. Perform all dialyzing steps at 4°C.

7. Centrifuge the sample 10 minutes at 14,000 x g and collect the supernatant containing DF or PP conjugated to HSA.
8. Check supernatant for proteins by performing a SDS-PAGE, using 12% gels. Load about 12 µg of HSA conjugate into one gel slot.

## 2. Determine coupling rate of drug conjugates (Figure 2)

1. For the analysis of the coupling rate of DF and PP conjugates by HPSEC use a 100 mM sodium phosphate buffer pH 6.5 containing 150 mM NaCl and 0.05% sodium azide. Use a 7.8 x 300 mm TSK-Gel-G2000<sub>SWXL</sub> column.
2. Perform detection of signals by using an online-coupled RI and UV detector system.
3. Prepare a HSA standard sample with a concentration of 1 mg/ml in dH<sub>2</sub>O. For detector calibration inject 50 µl HSA standard solution.
4. Calculate the RI constant  $k_{RI}$  and the UV constant  $k_{UV}$  for the detector. Use the formulas  $area_{RI}=k_{RI} \cdot [(dn/dc)_{HSA} \cdot c(HSA)]$  and  $area_{UV}=k_{UV} \cdot [(dA/dc)_{HSA} \cdot c(HSA)]$  with  $(dn/dc)_{HSA}=0.185$  and  $(dA/dc)_{HSA}=0.518^5$ .
5. Prepare DF and PP derivative standards for HPSEC that 5, 10, and 30 nmol amounts of both standards can be easily injected.
6. Calculate mean values for  $(dn/dc)_{drug}$  and  $(dA/dc)_{drug}$  for both standards. For DF, a  $(dn/dc)$  of  $0.235 \pm 0.010$  and a  $(dA/dc)$  of  $39.000 \pm 0.493$  was determined. For PP, a  $(dn/dc)$  of  $0.328 \pm 0.016$  and a  $(dA/dc)$  of  $53.155 \pm 2.464$  was determined.
7. Inject drug conjugates on HPSEC and determine their RI and UV peak areas.
8. Calculate the concentration of DF, PP derivative, and HSA by solving the two equations  $area_{RI}=k_{RI} \cdot [(dn/dc)_{drug} \cdot c(drug) + (dn/dc)_{HSA} \cdot c(HSA)]$  and  $area_{UV}=k_{UV} \cdot [(dA/dc)_{drug} \cdot c(drug) + (dA/dc)_{HSA} \cdot c(HSA)]$  for the unknowns.

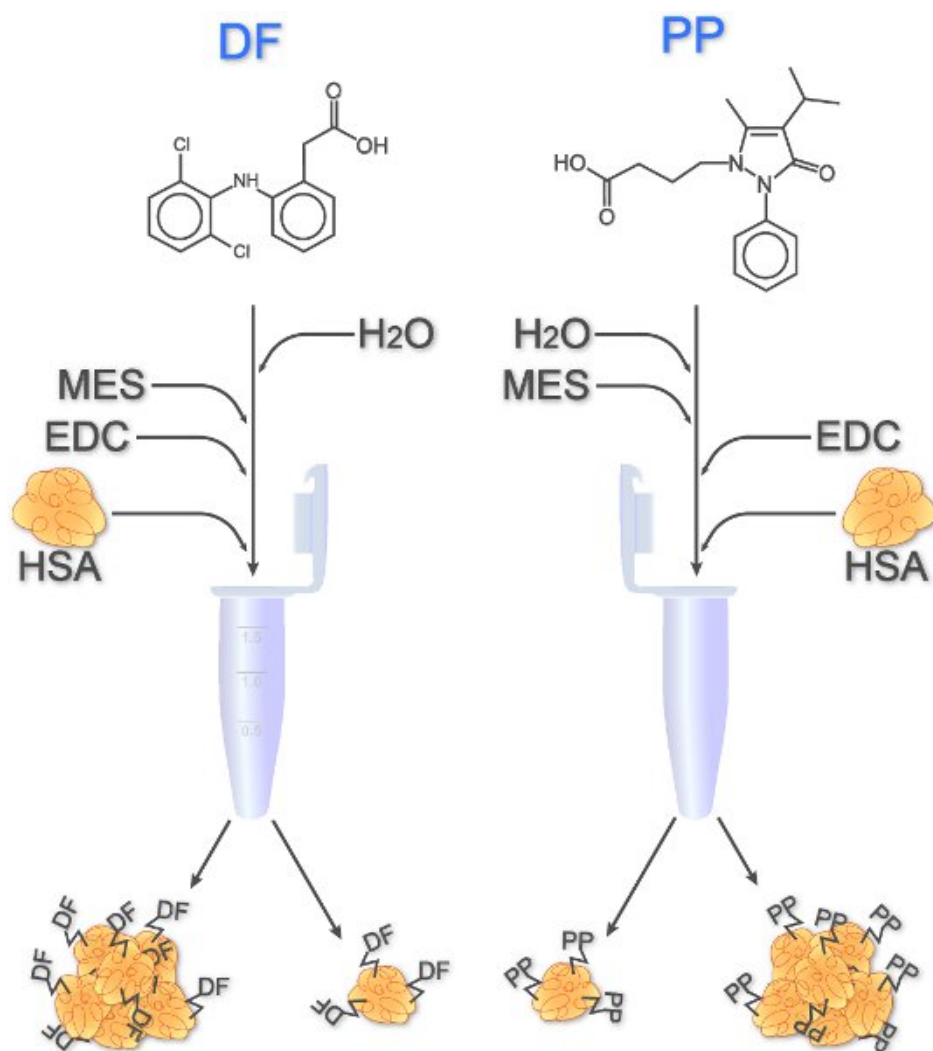
## 3. BAT using drug conjugates

1. Prepare seven flow cytometry vials and label them depending on their content: unstained control, negative control, positive control, and drug conjugates.
2. Pipette 50 µl of B-CCR-STB stimulation buffer (Flow2 CAST, Bühlmann Laboratories, Schönenbuch, CH) into the vials reserved for the unstained and negative control. As a positive control, use 50 µl of an anti-FcεRI solution provided by the Flow2 CAST kit. Add the appropriate amounts of conjugate solution to the vials reserved for the drug conjugates by creating a 1:10 dilution series from 0.02 -20 µg/ml DF conjugate and PP conjugate (final concentrations in an assay volume of 200 µl). These titration experiments must be performed to determine the concentration of optimal basophil activation for each patient sample.
3. Add 100 µl stimulation buffer to each tube and 50 µl patient's EDTA whole blood. Mix the samples carefully.
4. Pipette 20 µl Flow2 CAST staining reagent containing anti-CCR3 antibodies labeled with phycoerythrin (PE) and anti-CD63 antibodies labeled with fluorescein isothiocyanate (FITC) to each tube and mix again. Do not pipette staining reagent into the unstained sample!
5. Incubate the samples for 45 minutes in a 37°C water bath.
6. Stop reaction on ice for 5 minutes.
7. Lyse erythrocytes by adding 2.0 ml Flow2 CAST lysing reagent to each vial and vortex gently. Incubate samples in the dark for 10 minutes at room temperature.
8. Centrifuge samples for 5 minutes at 500 x g and carefully decant supernatant.
9. Resuspend cells in 500 µl Flow2 CAST wash buffer and store on ice until flow cytometry.
10. Adjust the flow cytometer's voltage settings for forward and side scatter (FSC, SSC) while acquiring the unstained sample.
11. Gate the cells predicted as lymphocytes from the SSC-FSC plot to the SSC-PE plot. Basophils are identified by a PE-labeled anti-CCR3 antibody as CCR3<sup>hi</sup> SSC<sup>lo</sup> and gate them into the FITC-PE plot. The anti-CD63 antibody detecting activated basophils is labeled with FITC.
12. Set the cut-off to maximum 2.5% of basophils as CD63<sup>hi</sup> in the negative control. A sample is considered positive for basophil activation if >5% basophils are CD63<sup>hi</sup> and the mean fluorescence index (MFI) of the sample divided by the MFI of the negative control exceeds 2 (Figure 3).

## 4. Representative Results:

This experiment evaluates BAT as a beneficial tool to detect IgE-dependent drug hypersensitivity. Protein conjugates of NSAIDs are used for activation of basophils. By HPSEC with RI and UV detection aggregation state, coupling degree, and effective yield of the conjugates are determined. As Figure 4 shows, 43.5% of the DF conjugates remained monomeric with a coupling degree of 5.0 DF/HSA. For the aggregates a coupling degree of 9.5 was determined. The propyphenazone conjugate was shown to consist of 68.5% monomers with a coupling degree of 21.2. The coupling degree of the aggregates was 27.9. In total, the determined coupling degree of DF conjugates was 7.6 DF and that of PP conjugates was 23.2.

BAT performed with conjugates under optimized conditions (concentration of conjugates, stimulation time) allowed investigation of IgE-dependent reactions in NSAID hypersensitivity. As shown in Figure 5, only the PP conjugate was able to trigger basophil activation visualized by an upregulation in CD63 surface expression: 20.6% of basophils were activated characterized by a log-shift in fluorescence intensity. The MFI increased by a factor of 4.9 from 386 (negative control) to 1893. In contrast, using the DF conjugate only 3.1% of the basophils were activated which was below the 5% cut-off. Also the MFI increased only by a factor 1.1 (limit 2.0) from 197 (negative control) to 210. Assay validity is given by (i) <5% basophil activation in the negative control, (ii) a log-shift in fluorescence intensity of a basophil subpopulation, and (iii) >50% activated basophils (positive control).



**Figure 1.** Coupling of DF and PP derivative to HSA. After dissolving the drugs, water, MES buffer, and EDC (coupling reagent) are added. The samples are vortexed for 1 minute before HSA is pipetted to the drug solutions. Within 2 hours shaking at room temperature the drugs covalently bind to HSA. Monomers as well as aggregates may result.

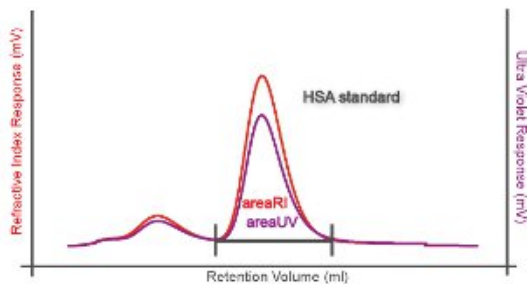
### 1. Calculate constants $k_{RI}$ and $k_{UV}$

given:  
 $(dn/dc)_{HSA}$   
 $(dA/dc)_{HSA}$   
 $c(HSA)$

determined:  
 peak  $area_{RI}$   
 peak  $area_{UV}$

$$area_{RI} = k_{RI} * [(dn/dc)_{HSA} * c(HSA)]$$

$$area_{UV} = k_{UV} * [(dA/dc)_{HSA} * c(HSA)]$$



### 2. Calculate mean $(dn/dc)_{drug}$ and $(dA/dc)_{drug}$

given:  
 $k_{RI}$   
 $k_{UV}$   
 $c(drug)$

determined:  
 peak  $area_{RI}$   
 peak  $area_{UV}$

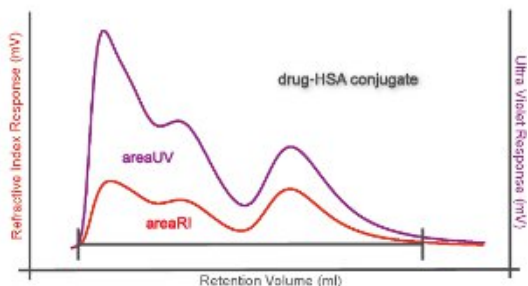
$$area_{RI} = k_{RI} * [(dn/dc)_{drug} * c(drug)]$$

$$area_{UV} = k_{UV} * [(dA/dc)_{drug} * c(drug)]$$

### 3. Calculate $c(drug)$ and $c(HSA)$

given:  
 $k_{RI}$   
 $k_{UV}$   
 $(dn/dc)_{drug}$   
 $(dA/dc)_{drug}$   
 $(dn/dc)_{HSA}$   
 $(dA/dc)_{HSA}$

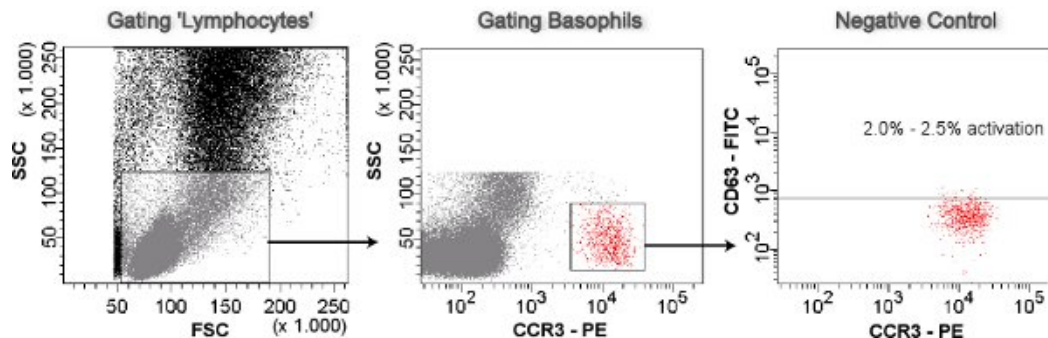
determined:  
 peak  $area_{RI}$   
 peak  $area_{UV}$



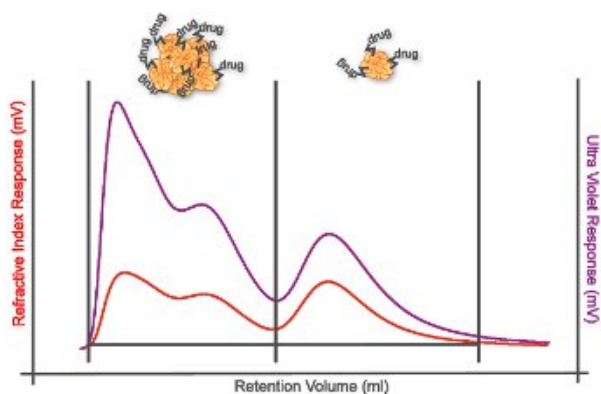
$$area_{RI} = k_{RI} * [(dn/dc)_{drug} * c(drug) + (dn/dc)_{HSA} * c(HSA)]$$

$$area_{UV} = k_{UV} * [(dA/dc)_{drug} * c(drug) + (dA/dc)_{HSA} * c(HSA)]$$

**Figure 2.** Calculation of coupling degrees. First, the constants  $k_{RI}$  and  $k_{UV}$  are determined. Therefore, HSA standard with known concentration is injected into the HPSEC system.  $(dn/dc)$  and  $(dA/dc)$  of HSA are given values of 0.185 and 0.518, respectively<sup>5</sup>. The peak areas are used for calculating the constants. Second, for each drug three standards are injected to determine drug- and concentration-specific RI and UV areas. Since the  $k_{RI}$  and  $k_{UV}$  constants are known from the steps above, the drug-specific  $(dn/dc)$  and  $(dA/dc)$  derivatives can be calculated. Third, drug-HSA conjugates are injected into HPSEC. The resulting peak areas are used to calculate the concentrations of drug and HSA per peak by solving the two equations with two unknown variables.

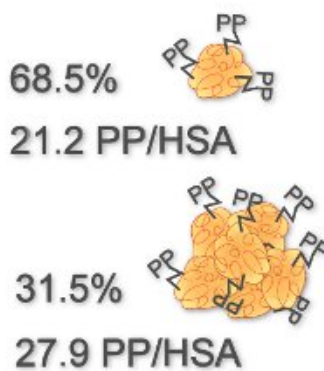
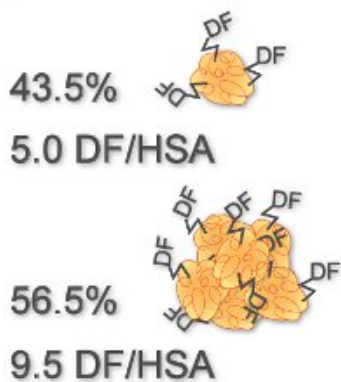


**Figure 3.** Basophil gating. Cells predicted as lymphocytes are gated side scatter versus forward scatter (SSC-FSC plot). The basophils are identified as  $CCR3^{hi} SSC^{lo}$  from the gated lymphocytes (SSC-CCR3-PE plot). Basophils are analyzed for activation ( $CD63^{hi}$ ) in the CD63-FITC-CCR3-PE plot. The negative control is used to set the cut-off of maximum 2.5% basophils remaining  $CD63^{hi}$ .



DF

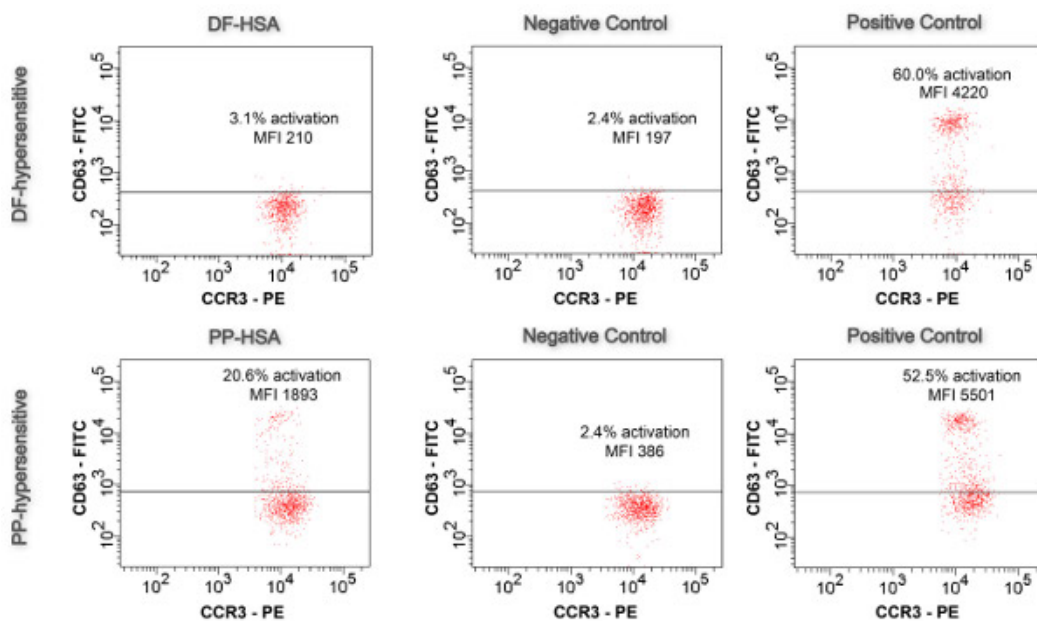
PP



total 7.6 DF/HSA

total 23.2 PP/HSA

**Figure 4.** Determined coupling degrees of drug conjugates. RI and UV signals show two peaks representing aggregates (eluting at low retention volume) and monomers (eluting at high retention volume) of drug-HSA conjugates. Percentages of monomers and aggregates (determined from RI signals) and their coupling degrees are depicted (n=1).



**Figure 5.** Basophil activation test. Results of drug conjugate-activated samples, negative controls, and positive controls are shown in CD63-FITC-CCR3-PE plots (n=1).

## Discussion

BAT is a well-established although not yet routinely used method for diagnosis of IgE-mediated allergic disease<sup>6,7</sup>. For drug hypersensitivity, however, its applicability is compromised as low molecular weight compounds are not able to crosslink IgE receptors, a prerequisite for basophil activation<sup>8</sup>. Therefore, drugs under investigation need to be covalently coupled to suitable carrier proteins (e.g. HSA). Importantly, the coupling degree (i.e. number of drug molecules per carrier protein) needs to be controlled to guarantee immunological activity (i.e. IgE receptor cross-linking) of conjugates. Theoretically, two haptens per carrier molecule should be sufficient for IgE receptor cross-linking and as few as five DF molecules per HSA have been shown to trigger mediator release in a cell-based assay<sup>4</sup>. Both conjugates, PP-HSA and DF-HSA, have been determined to display a sufficiently high coupling degree (HPSEC) and to be immunologically active making them suitable reagents for use in BAT. Another issue may be that drug metabolites may play a role, as a metabolite instead of the parent drug may cause the hypersensitivity reaction. In case of DF, this possibility has been evaluated in detail previously using five major Phase I metabolites and one linkage variant<sup>4</sup>. Important factors for mastering the described technique include quality of blood sample (<12 hours since blood collection, number of detected basophils >500) and optimization of stimulation conditions (time, dose). In addition, so-called non-responders who do not even react with the positive control (antibody directed against the IgE receptor) have to be identified. Therefore, validation criteria have to include an anti-FcεRI antibody as positive control. An important advantage of using drug conjugates is the fact that they appear non-toxic in contrast to pure drugs, as shown for DF-HSA conjugate by co-stimulation with anti-FcεRI antibody in BAT<sup>4</sup>. Pure DF, in contrast, displays cytotoxic effects at a concentration of 1.25 mg/ml causing problems with the interpretability of BAT<sup>9</sup>. Generally, testing for potential cytotoxicity is strongly recommended for every newly produced drug conjugate.

Here, we have shown the potential of PP-HSA conjugate used in BAT to reveal IgE-mediated hypersensitivity. In contrast, DF hypersensitivity is not associated with IgE demonstrated by the lack of basophil activation in response to DF-HSA conjugate. Importantly, in case of uncertainty concerning an IgE-mediated mechanism, conjugates have to be evaluated for immunological activity by *in vitro* cell-based assay systems as has been shown for DF<sup>4</sup>.

Using the described setup of conjugating low molecular weight drugs covalently to suitable protein carrier molecules a number of hypersensitivity reactions against drugs including antibiotics, other NSAIDs, radiocontrast media, muscle relaxants, anesthetics, etc. may be investigated for an involvement of an IgE-mediated mechanism. Hence, BAT may serve as an addition to the existing methods for diagnosis (skin test, oral provocation testing).

## Disclosures

No conflicts of interest declared.

## Acknowledgements

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## Ethics statement:

The study was approved (PLUS\_Ethik\_090514) by the Ethics Committee for Experiments Involving Humans and/or Animals at the University of Salzburg complying with the Helsinki Declaration as revised in 1983 and all patients participating gave their written informed consent.

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