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Basophil activation test in the diagnosis of drug-induced hypersensitivity reactions: a retrospective study of Slovak patients

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Key words

Adverse drug reactions; basophil activation test; CD63 marker; drug hypersensitivity; flow cytometry.

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Impact statement

BAT is a useful complementary tool for diagnosing DHRs, predicting and monitoring clinical responses to treatment and searching potentially non-cross-reactive alternatives.

Summary

Background. *Drug hypersensitivity reactions (DHRs) include allergic, exaggerated pharmacologic, and pseudoallergic reactions to medications. The basophil activation test (BAT) using CD63 expression is a sensitive and specific complementary tool in the diagnosis of immediate DHRs.* ***Methods.*** *In this study we analyzed retrospectively 1,160 patients with history of DHRs categorized into several subgroups of diagnoses. BAT using CD63 expression was performed in the whole blood and results were analyzed by flow cytometry.* ***Results.*** *The mean value of CD63 expression was significantly higher for positive patients than negative/borderline findings ($p < 0.001$). The highest frequencies of positive BAT were in categories of diagnosis codes D89 (other disorders involving the immune mechanism, not elsewhere classified) and L50 (urticaria). The percentage values were higher in the group of antibiotics and anticoagulants. Comparing individual drugs in vitro, we observed the highest prevalence of positive reactions by local anesthetics (Mepivastesin, Mesocain, Lidocaine), antibiotics (Amoxiclav, Megamox duo), antirheumatics, antiphlogistics and antiuratics (Ibuprofen, Ibalgin). The BAT positivity decreases with age, there were significant differences in the CD63 activation between old age and children, younger adults, and older adults ($p < 0.001$, $p < 0.05$ and $p < 0.01$, respectively). No significant differences have been found between men and women.* ***Conclusions.*** *BAT is a useful complementary tool to support the diagnosis of drug hypersensitivity conditions, especially in cases with severe reaction where drug challenge is contraindicated. The implementation of BAT in clinical practice is valuable in the workup of DHRs and extremely useful in the case of life-threatening drug allergies.*

Introduction

Adverse drug reactions (ADRs) are an important public health problem and can be life-threatening. ADRs are classified into two main types: type A reactions, which are dose dependent and predictable. These kinds of reactions constitute 70-80% of ADRs, and type B – which are unpredictable – include idiosyncrasy, drug intolerance, or drug allergy, and may comprise approximately 10-15% of all ADRs. Drug hypersensitivity reactions (DHRs) are unpredictable ADRs. Depending on the time passed between the consumption of the drug and symptom onset, reactions are classified as suggestive of either immediate hypersensitivity (0-6 h) or non-immediate hypersensitivity (hours-days). Drug allergy pathogenesis and clinical manifestations may vary depending on the culprit drug. The optimal approach to the drug allergy should be based on the risk stratification, the phenotype of hypersensitivity reaction, drug class, and patient's clinical needs. Hypersensitivity reactions are divided into 4 types (I-IV) by the Gell and Coombs classifi-

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cation. The hypersensitivity Type I reactions follow very quickly after contact with the allergen, generally between a few seconds to 30 minutes (immediate type). Allergic rhinitis and conjunctivitis, allergic asthma, acute urticaria and IgE-mediated anaphylaxis belong to this type. The response times of types II and III hypersensitivity reactions are slower than that of type I reactions; they typically develop 3-6 h after exposure to antigen. The fourth type is considered a delayed hypersensitivity reaction because it usually occurs more than 12 hours after exposure to the allergen, with a maximal reaction time between 48 and 72 hours. Type IV hypersensitivity reactions occur because of T cell response to an antigen leading to an inflammatory response. These reactions are further subdivided (IVa through IVd) based on the type of T cells involved. Common symptoms of type IV hypersensitivity include red, itchy, painful rash with blisters, or patches of dry, cracked, scaly skin. The clinical presentation is based on the distinct condition that develops. Contact dermatitis is a very common type IV hypersensitivity reaction. Symptoms without demonstration of being an immunological process are classified as non-immune DHRs, and they are generally related to nonspecific histamine release, nonspecific complement activation, bradykinin accumulation or induction of leukotriene synthesis in type I hypersensitivity reactions. Non-steroidal anti-inflammatory drugs and antibiotics are most often implicated in drug allergies, besides anesthetic drugs, latex, insulin, and immunomodulators (1-3).

The mechanisms underlying immunogenic-mediated DHRs involve the adaptive immune system and comprise both IgE- and non-IgE-mediated mechanisms. In contrast, nonimmunogenic-mediated DHRs mechanisms do not involve the adaptive immune system. The diagnosis of immune mediated DHRs is based on clinical history, immediate-reading skin tests and provocation tests. Skin prick testing (SPT) provides evidence for sensitization and can help to confirm the diagnosis of a suspected type I allergy, the intradermal test (IDT) is used for both immediate and delayed hypersensitivity and may be used when soluble forms of the drugs are available. Skin tests are the most used procedure to confirm a sensitization in drug hypersensitivity, they have high specificity, but unfortunately, the sensitivity of skin tests to most drugs is low (SPT 49% and IDT 73%, respectively) (4, 5). SPT is widely practiced, carries very low (but not negligible) risk of serious side effects to patients. The overall risk of inducing anaphylactic reactions by SPT is less than 0.02%, whereas it is slightly increased if drug, food, latex, or hymenoptera venom extracts are used. IDT has an increased antigen load to which the body is exposed through the skin, and is associated with a higher risk of systemic reaction than SPT. There are limitations to perform skin tests, *e.g.* discontinuation of some medications before testing, age, needle aversion, serious risk of anaphylaxis, skin disease. Therefore, in cases of negative reactions, drug allergies cannot be excluded. The evaluation of DHRs involves three main strategies: accurately reviewing the patient's clinical history, conducting diagnostic tests (skin tests and/or *in vitro* tests) and performing drug challenge tests. Challenge tests are currently the gold standard for diagnosis, but they often involve significant risks, especially in patients who have received multiple drugs in the context of an adverse reaction and/or patients with multiple comorbidities. In the evaluation, a careful investigation of medical history and laboratorial parameters is extremely important. *In vitro* tests can be used to support this process, and may be considered as an alternative, particularly in cases with a history of a life-threatening reaction. Tests require specialized equipment and trained personnel and thus are not broadly available. It is essential to construct networks of specialized centers to expand the knowledge of these techniques and to adequately validate them in as many centers as possible (6-10).

The basophil activation test (BAT) is a cell-based functional assay for the detection of basophils in whole blood following allergen stimulation using flow cytometry. The BAT evaluates activation markers (CD63 and/or CD203c) expressed on the cell membrane of basophils. CD63 is a membrane-bound molecule, intragranularly expressed, and exposed on the cell surface during degranulation where granular membrane fuses with the cellular membrane. Basophils constitute a minor fraction – less than 1% of the circulating white blood cells – and upon IgE cross-linking by antigen, can activate and degranulate. They expel the preformed content from their granules, as well as *de novo* synthesized mediators. Degranulation of basophils can also result from other mechanisms, such as non-IgE-mediated pathways (*e.g.* cytokines, anaphylatoxins, IgG) and/or direct activation (*e.g.* opioids, iodinate contrast media). BAT could be a predictive test evaluating CD63 as a biomarker of basophil activation for the assessment of immediate DHRs. BAT is more sensitive and specific than other *in vitro* diagnostic techniques in drug allergy and is essentially complementary to skin tests and allergen-specific IgE determinations. Increasing the number of diagnostic tests used to confirm a suspected clinical history of allergy can improve diagnosis efficiency and accuracy. BAT reduces the need for *in vivo* procedures, such as intradermal tests and allergen challenges, which can cause allergic reactions of unpredictable severity. It is a very important diagnostic tool, less invasive, more comfortable and less expensive. The main objective in the management of a DHRs is to search for the culprit drug to order instruct future avoidance, when possible, and to clarify tolerance to alternative treatments for the patient (11-13).

The aim of this research is to explore retrospectively a cohort of patients recruited from 2020 to 2023 at diagnostic Immunology Laboratory of Slovak Medical University in Bratislava and tested to DHRs. We evaluated BAT, the expression of activation marker CD63, and confounders such as age, gender, diagnostic category code and drug class. BAT not only complement culprit identification but can also be used to test potentially non-cross-reactive alternatives, thereby avoiding drug challenge tests.

Materials and methods

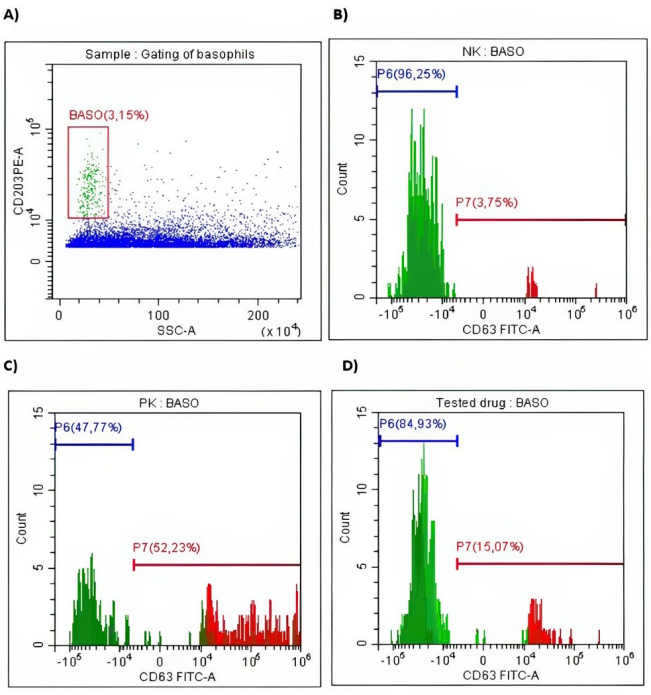
Study population

We performed a cohort study assessing DHRs on collected clinical data from 1,160 patients (876 women and 284 men). Some patients were tested *in vitro* for more than one type of drug. A total of 3,223 laboratory tests were evaluated. The study was retrospective and did not require any interaction with the patients. Ethical approval was waived by the local Ethics Committee of the Slovak Medical University in view of the retrospective nature of the study and all the procedures being performed were part of the routine care. The participants were referred to Polyclinic of Slovak Medical University (SMU Polyclinic) after detailed anamnesis and allergological workup performed by clinical allergists/immunologists and dermatologists from Bratislava and surroundings over the years 2020 to 2023. The blood sampling was provided in the collection room of SMU Polyclinic and BATs were processed in the diagnostic Immunology Laboratory. The patients were instructed to stop taking antihistamines, antileukotrienes and glucocorticoids at least 48 hours before sampling. Antihistamines and antileukotrienes do not interfere with BAT results, the oral administration of these pharmacological substances may affect others cell function assays that we performed (*e.g.* lymphocyte activation and proliferation – submitted). Blood samples were drawn from patients at least 2-4 weeks post-hypersensitivity reaction, collection of whole blood was done in heparin and performed within 4 h of blood collection to maximize viability and functionality of basophils.

Basophil activation test (BAT)

For the quantification of immediate DHRs was used BAT, that analyses expression of basophil activation marker, CD63. Our study was focused on the basophil population at a single cell level using flow cytometry. This technique allowed the specific examination of basophils in whole blood, that is more physiological and more closely resemble the *in vivo* environment of blood basophils, than isolated basophils. Heparinized blood was incubated with or without relevant drugs. The optimization procedure consisted of standardization of drug concentrations, using two drug concentrations, dilutions were prepared shortly before BAT was performed. Analysis of the CD63 antigen on basophils was examined using the BasoFlowEx kit (Exbio, Prague, Czech Republic) with a modification for the lyse-no-wash procedure (14). Briefly, tubes were prepared for negative control, positive control (stimulation control), and samples stimulated with different drug substances. We tested the common commercially available drugs used in therapy. The pharmaceuticals were in the form of tablets, or injectable preparations. Stock solutions were produced by pulverizing if necessary and dissolving the drug in 10 ml of sterile isotonic saline (NaCl 0.9%), after checking concentration of drug solubility in aqueous solutions. The concentrations of stock solutions were based on active substance content of the concerned medicine. In the test, we used two drug dilutions, or more as needed, to find conditions in which cell activation and survival outweigh cell death. For example, the range of final concentration was 0.25-2.5 mg/ml (Amoxicillin and Clavulanic Acid), 0.2-2.0 mg/ml (Metamizole sodium salt, Paracetamol), 0.15-1.5 mg/ml (Ibuprofen), 0.1-1.0 mg/ml (Mepivacaine hydrochloride), 0.08-0.8 (Lidocaine hydrochloride), 0.04-0.4 mg/ml (Trimecaine hydrochloride, Propofol), 0.45-4.5 mg/ml (Nadroparin calcium salt). For hypolipidemic category (*e.g.* Atorvastatin, Simvastatin, Fluvastatin) the range 0.01-0.3 mg/ml was used. Stimulation Buffer and heparinized blood were added into all tubes and incubated at 37 °C for 15 minutes. Staining reagent (antibody cocktail: anti-CD63-FITC + anti-CD203c-PE) was added, and samples were incubated for 20 minutes at 2-8 °C. The red blood cells were lysed by lysing solution OptiLyse C (Beckman Coulter, Marseille, France) and samples were analyzed using clinical flow cytometers (Cytomic FC500 and DxFLEX, Beckman Coulter) within two hours after staining. Enough basophils for analysis were > 200. Histograms of negative control and positive control samples, and drug-stim-

Figure 1 *- Example of BAT histograms (negative control, positive control, and drug-stimulated basophils).*



BAT: basophil activation test. **(A)** Gating of basophils: basophils were identified as CD203+ cells; **(B)** Negative control sample, CD63 negative threshold (P7 = 3.75 %); **(C)** Positive control sample, value of ≥ 15% activated basophils (P7 = 52.23 %); **(D)** Drug-stimulated sample, CD63 positivity: the cut-off value ≥ 5% and stimulation index (SI) ≥ 2 (P7 = 15.07 %).

ulated basophils are shown in **figure 1**. We have calculated the percentage of activated basophils (CD63+), and individuals are considered positive when the percentage of CD63+ exceeds the cut-off value ≥ 5% and stimulation index (SI) ≥ 2. The limit of borderline range (suspect zone) was < 5% and SI ≥ 2. If the positive control sample exhibited a value of ≥ 15 activated basophils, the samples could be evaluated. A positive control sample was stimulated using a monoclonal antibody against IgE molecule which mimics the allergen crosslinking process of IgE molecules on the basophil surface and using a chemotactic peptide N-formyl-Met-Leu-Phe (fMLP). The fMLP activates basophils through the G-protein coupled fMLP receptors and is often used as a non-IgE-mediated positive control. The main limitation of BAT was the patient with nonresponder basophils (basophils that do not respond to stimulants), rendering BAT uninterpretable. Nonresponder and patients with low basophil counts were excluded from the study (6%). Non-responsiveness is attributed to disturbances in the signalosome of the FcεRI pathway, particularly failure to express the downstream tyrosine kinase Syk (15, 16). In case of positive BAT, it is not necessary to perform a provocation test. Negative results could not rule out a suspected drug, they may help the allergist manage and ensure availability of appropriate drugs for further *in vivo* test or drug provocation test. A no-positive answer indicates that patients may not actually be allergic to the suspected drug or have the chance to use alternative drugs.

Statistical analysis

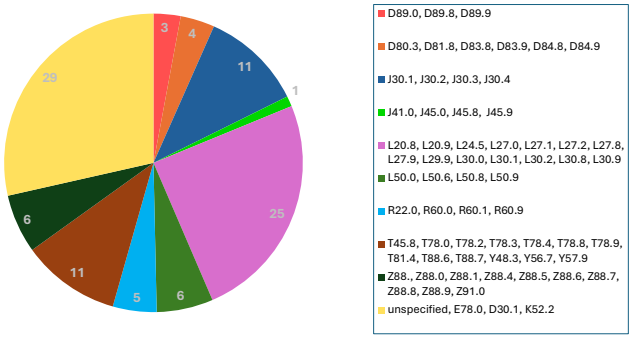
BAT data was tested for normalized distribution and analyzed using the Mann-Whitney test. The expression of CD63 was described as median (50th percentile), and statistical significance was defined $p < 0.05$. The median was calculated using descriptive statistics. All statistical analyses were conducted using SPSS Statistics, version 27 (IBM, USA). The subjects with unresponsive basophils were eliminated from data analysis.

Results

Subjects were referred to diagnostic testing after experiencing a drug-induced hypersensitivity reaction. The time elapsed between the reaction and the performance of the test differed in patients, on average 3-5 months. The most common clinical symptoms were skin reaction, pruritus, rash, urticaria, redness, vomiting, heart palpitations, swelling of the face, tongue, difficulty breathing and swallowing, anaphylactic reaction, collapse state. Detailed and complete description of clinical symptoms by doctors not stated for every patient. Results of skin prick tests or specific IgE were not available for those who performed BAT. Patients were divided into several groups based on clinical symptoms (*e.g.* cutaneous, respiratory, gastrointestinal, cardiovascular, hypotension, confusion), in which we monitored changes in CD63 expression and frequency of BAT positivity.

We most often tested the diagnoses indicated by the codes of International Classification of Diseases (ICD) (17): L20-L30 (dermatitis and eczema), J30 (vasomotor and allergic rhinitis), T78 (adverse effect, not elsewhere classified), Z88 (personal history of allergy to drugs, medicaments and biological substances), L50 (urticaria) and group of patients with an unspecified diagnosis. To process the results, we categorized the probands into ten subgroups of diagnoses (**figure 2**). The group of dermatitis and eczema (25% of the total) includes subgroups: L20.8 - other atopic dermatitis, L20.9 - atopic dermatitis, unspecified, L24.5 - irritant contact dermatitis due to other chemical products, L27.0 - generalized skin eruption due to drugs and medicaments, L27.1 - localized skin eruption due to drugs and medicaments, L27.2 - dermatitis due to ingested food, L27.8 - dermatitis due to other substances taken internally, L27.9 - dermatitis due to unspecified

Figure 2 *- Percentage distribution of patients according to subgroups of diagnoses (ICD codes).*



ICD: International Classification of Diseases; World Health Organization (2019). International statistical classification of diseases and related health problems (11th ed.). Available at: https://icd.who.int/.

D89 - Other disorders involving the immune mechanism, not elsewhere classified. D80 - Immunodeficiency with predominant antibody defects. D81 - Combined immunodeficiencies. D83 - Common variable immunodeficiency. D84 - Other immunodeficiencies. J30 - Vasomotor and allergic rhinitis. J41 - Simple and mucopurulent chronic bronchitis. J45 – Asthma. L20 - Atopic dermatitis. L24 - Irritant contact dermatitis. L27 - Dermatitis due to substances taken internally. L29 – Pruritus. L30 - Other dermatitis. L50 - Urticaria. R22 - Localized swelling, mass and lump of skin and subcutaneous tissue. R60 - Edema, not elsewhere classified. T45 - Poisoning by primarily systemic and hematological agents, not elsewhere classified. T78 - Adverse effects, not elsewhere classified.

T81 - Complications of procedures, not elsewhere classified. T88 - Other complications of surgical and medical care, not elsewhere classified. Y48 - Anesthetics and therapeutic gases. Y56 - Topical agents primarily affecting skin and mucous membrane and ophthalmological, otorhinolaryngological and dental drugs. Y57 - Other and unspecified drugs and medicaments. Z88 - Personal history of allergy to drugs, medicaments and biological substances. Z91 - Personal history of risk-factors, not elsewhere classified. E78 - Disorders of lipoprotein metabolism and other lipidemias. D30 - Benign neoplasm of urinary organs. K52 - Other noninfective gastroenteritis and colitis.

substance taken internally, L29.9 - pruritus, unspecified, L30.0 - nummular dermatitis, L30.1 - dyshidrosis (pompholyx), L30.2 - cutaneous autosensitization, L30.8 - other specified dermatitis, L30.9 - dermatitis, unspecified. The group of vasomotor and allergic rhinitis (11 % of the total) included J30.1 - allergic rhinitis due to pollen), J30.2 - other seasonal allergic rhinitis, J30.3 - other allergic rhinitis, J30.4 - allergic rhinitis, unspecified. The group of patients with lower airway diseases J41.0 - simple chronic bronchitis, J45.0 - predominantly allergic asthma, J45.8 - mixed asthma, J45.9 - asthma, unspecified was represented by only 1%. The age distribution of patients was children (1-17 years; 8%), younger adults (18-44 years; 29%), older adults (45-64 years; 36%), and old age (65-99 years; 27%).

We compared the CD63 marker obtained by flow cytometry analysis between groups: a) negative response or borderline (suspect zone) and b) positive response to a drug. The distribution data were represented as percentile values (Q1 = 5.9; Q2 = 7.8; Q3 = 13.2). The median value of CD63 expression in positive BAT response was significantly higher than in negative or borderline response (1.60 %) ($p < 0.001$). Of the total number of BAT, we recorded about 10% positive drug responses (n = 3,223). BAT is expected to be positive for reactions that are clinically type I, this functional test measures the degree of basophil degranulation following allergen stimulation. In the case of BAT negativity, there may be another type of hypersensitivity reaction, a false negativity, or another mechanism, such as non-IgE mediated or direct basophil activation.

The frequency of BAT positivity decreases with age. The prevalence of positive drug responses was as follows: children 23.4% of 154 tests, younger adults 10.4% of 838 tests, older adults 9.2% of 1,230 tests, and the elderly 7.6% of 1,001 tests. We did not observe significant differences in the response to stimulants in the positive control between age groups. The difference in frequency of positive basophil activation between men (11.8% of 738 tests) and women (9.1% of 2,485 tests) was not significant. Patients with diagnoses a) D89.0 (polyclonal hypergammaglobulinemia), D89.8 (other specified disorders involving the immune mechanism, not elsewhere), D89.9 (disorder involving the immune mechanism, unspecified), and b) L50.0 (allergic urticaria), L50.6 (contact urticaria), L50.8 (other urticaria), L50.9 (urticaria, unspecified) represented the highest frequency of positive BAT (13.8% of 94 tests, and 12.6% of 167 tests, respectively) (**table I**).

The most frequently tested drug classes were anesthetics and antibiotics, as shown in **figure 3**. The highest frequency of BAT positivity was for antibiotics (16.2% of 464 tests) and anticoag-

Table I *- Frequency of BAT positive responses.*

Diagnosis (ICD codes classification)	Frequency of BAT positivity (%)	Total number of BAT
D89 - Other disorders involving the immune mechanism, not elsewhere classified	**13.8**	94
D80 - Immunodeficiency with predominantly antibody defects, D81 - Combined immunodeficiencies, D83 - Common variable immunodeficiency, D84 - Other immunodeficiencies	7.3	96
J30 - Vasomotor and allergic rhinitis	9.5	380
J41 - Simple and mucopurulent chronic bronchitis, J45 – Asthma	11.4	44
L20 - Atopic dermatitis, L24 - Irritant contact dermatitis, L27 - Dermatitis due to substances taken internally, L29 - Pruritus, L30 - Other dermatitis,	9.1	821
L50 - Urticaria	**12.6**	167
R22 - Localized swelling, mass and lump of skin and subcutaneous tissue, R60 - Edema, not elsewhere classified	10.1	159
T45 - Poisoning by primarily systemic and hematological agents, not elsewhere classified, T78 - Adverse effects, not elsewhere classified, T81 - Complications of procedures, not elsewhere classified, T88 - Other complications of surgical and medical care, not elsewhere classified, Y48 - Anesthetics and therapeutic gases, Y56 - Topical agents primarily affecting skin and mucous membrane and ophthalmological, otorhinolaryngological and dental drugs,Y57 - Other and unspecified drugs and medicaments	9.8	328
Z88 - Personal history of allergy to drugs, medicaments and biological substances, Z91 - Personal history of risk-factors, not elsewhere classified	6.2	210
E78 - Disorders of lipoprotein metabolism and other lipidemias, D30 - Benign neoplasm of urinary organs, K52 - Other noninfective gastroenteritis and colitis	10.2	924

Values in bold indicate the highest frequencies of positive BAT responses, which are further detailed in the text. ICD: International Classification of Diseases. World Health Organization (2019). International statistical classification of diseases and related health problems (11th ed.). Available at: https://icd.who.int/.

Figure 3 *- Drug classes frequently tested using BAT (%) (minimum number of laboratory tests ≥ 5%).*

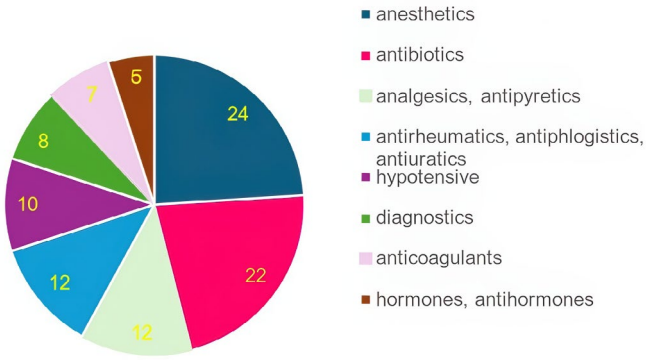
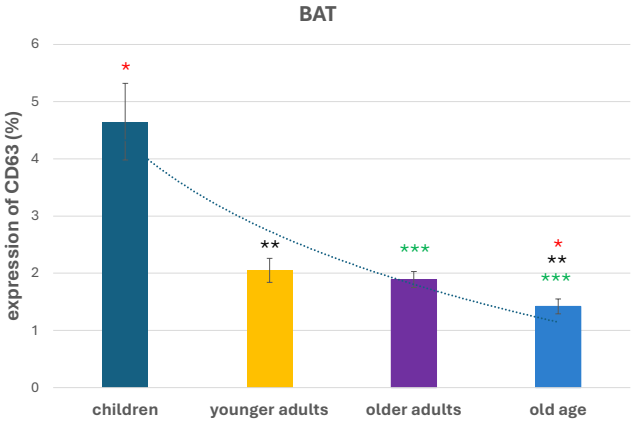


Figure 4 *- Distribution of CD63 expression in different age groups.*



BAT: basophil activation test. *Between old age and children, $p < 0.001$; **between old age and younger adults, $p < 0.05$; ***between old age and older adults, $p < 0.01$.

ulants (12.6% of 143 tests). Negative tests predominated in the classes of diagnostics (87.3% of 158 tests), hypotension (85.3% of 204 tests) and anesthetics (83.3% of 491). By comparing individual drugs, we found that the most frequent positivity of BAT *in vitro* belongs to 1) the antibiotics Amoxiclav, Megamox duo (Amoxicillin and Clavulanic acid), 2) among the local anesthetics Mepivastesin (Mepivacaine hydrochloride), Mesocain (Trimecaine hydrochloride), Lidocaine (Lidocaine hydrochloride), 3) the general anesthetic Propofol), 4) the class of antirheumatic drugs, antiphlogistics and antiuratics Ibalgin (Ibuprofen) and Ibuprofen, 5) anticoagulant Fraxiparine (Nadroparin calcium salt), and 6) analgesics, antipyretics Novalgin (Metamizole sodium salt) and Paralen (Paracetamol) (**table II**).

We recorded a positive response to at least two drugs in 4.5% of the total number of patients, 2% were drugs of the same group (especially antibiotics) and 0.25 % were drugs with the same substance.

Children, younger adults, and older adults had a high frequency of positive BAT responses to antibiotics. In old age, the positivity of hypolipidemic drugs dominated, but the drug representation for this category of drugs was very low (< 5% of total BAT). There were significant differences in CD63 expression between the elderly and other groups, namely children, younger adults, and older adults ($p < 0.001$, $p < 0.05$, and $p < 0.01$, respectively) (**figure 4**). CD63 expression was not significantly different between men and women.

The patients were analyzed also according to presenting symptoms, most of them presented more symptoms. There were no differences between clinical manifestations of drug allergies (*e.g.* cutaneous, respiratory, gastrointestinal, cardiovascular, hypotension) and CD63 marker expression or BAT positivity frequency. No significant differences were found when comparing the frequency of positive BAT between patients with and without anaphylactic reaction.

Table II *- Drugs with the most frequent BAT positivity.*

Commertial name of drug	Effective drug substance	Drug class
Amoxiclav, Amoxixlav syrup, Megamox duo	Amoxicillin and Clavulanic acid	Antibiotics
Mepivastesin	Mepivacaine hydrochloride	Local anesthetics
Mesocain	Trimecaine hydrochloride	Local anesthetics
Lidocain	Lidocaine hydrochloride	Local anesthetics
Propofol	Propofol	General anesthetics
Ibuprofen, Ibalgin	Ibuprofen	Antirheumatics, antiphlogistics, antiuratics
Fraxiparine	Nadroparin calcium salt	Anticoagulants
Novalgin	Metamizole sodium salt	Analgesics, antipyretics
Paralen	Paracetamol	Analgesics, antipyretics

Discussion and conclusions

DHRs induced by small molecule drugs include a broad spectrum of ADRs with heterogeneous clinical presentations and mechanisms. A high number of unconfirmed and/or self-reported DHRs is a frequent problem in daily clinical practice with impact on future prescription choices and patient health. The *in vitro* BAT can serve as a quick, reliable, and safe diagnostic tool, that can provide information about diagnostic algorithms for the management of DHRs. The utility of BAT is in patients with immediate DHRs, whom *in vivo* testing was considered high risk or when commercial antigens were not available (18, 19).

Assays which assess the activation of basophils by the changes in cell surface markers (CD63, CD203c) are now in routine use. CD203c has been identified as specific for basophils and is expressed on resting cells at low levels, and its expression is rapidly up-regulated following activation. The activation is transient and more rapid than expression of CD63, so assays which use only CD203c require careful consideration of the timing for detection, but the selectivity of this marker for basophils (among all circulating leukocytes) make this an attractive marker of activation (20). Several factors may influence the quality of the results, these include sampling conditions, relevant allergens, basophil-gating strategies, markers for detection of activated cells, drug concentrations, time that elapses between the reaction and blood sampling, medications used by the patient who is being tested. It is important for the clinician to understand the potential promise of *in vitro* tests for confirming or ruling out a diagnosis of DHRs. A positive response will lead to an increase in the probability that a diagnosis of DHRs is present and to subsequent drug avoidance. A negative test response should reduce the probability of an association between the test drug and DHRs and preclude avoidance of the suspect drug or further diagnostic testing. There is a need for characterization of a larger numbers of exposed control subjects (21, 22). DHRs are a burden for patients and health systems, not only due to the increasing prevalence (10-20% of hospitalized patients and up to 25 % of outpatients), but also to the complexity and severity of the reactions (23). Our study confirmed 1/10 of positive results from the total numbers of laboratory tests, CD63 expression in positive patients was significantly higher than in negative and/or borderline (suspicious) responses. Currently, several endophenotypic categories of DHRs are defined, such as type I IgE/non-IgE, cytokine release, mast-related G-protein coupled receptor X2 and cyclooxygenase-1 (COX-1). A proper knowledge of endotypes based on specific biomarkers will permit discriminating patients within the same phenotype. For diagnostic purposes, the fact that positive basophil responses cannot distinguish between sIgE-FcεRI cross-linking and alternative mechanisms is not a disadvantage. BAT can help to deepen our understandings in mechanistic endotypes of immediate DHRs, benefit identification of antibody recognition sites, expand our understanding of desensitization and tolerance induction strategies, and predict natural disease courses and prognosis. The mechanisms responsible for the immediate DHRs, without prior exposure and with low opportunity for prior sensitization to the drug, may raise hypothesis of non-IgE-mediated pathways, such as complement activation with release of anaphylatoxins, or direct activation of basophils and mast cells (24-26).

Anesthetics and antibiotics are the most common classes of drug tested in our laboratory. When comparing different drugs, we observed the highest frequency of positive results with antibiotics Amoxiclav, Megamox duo (Amoxicillin, Clavulanic acid), among local anesthetics Mepivastesin (Mepivacaine hydrochloride), Mesocain (Trimecain), Lidocaine (Lidocaine chloride monohydrate). Positive reactions *in vitro* also include general anesthetic Propofol, anticoagulant Fraxiparine (calcium salt of Nadroparin), antirheumatic, antiphlogistic and antiuratic drugs (Ibuprofen, Ibalgin - Ibuprofen) and analgesics, antipyretics (Novalgin - Metamizole sodium, Paralen - Paracetamol).

Most studies from allergy centers and clinics reported that the common culprit drugs among DHRs were antibiotics, radiocontrast media and antineoplastic drugs. Drug-specific cofactors for DHRs are diseases such as obesity and atopic dermatitis, knowledge of which may improve risk calculation in drug challenge trials (27-30). Amoxiclav is currently the most frequently reported beta-lactam in Europe. Another component, the Clavulanic acid, has been recently identified as prevalent triggers in perioperative anaphylaxis. The high positive predictive value of BAT to clavulanic acid shows its potential value as a complementary tool to the allergological workup of patients with immediate allergic reactions after amoxicillin-clavulanic acid treatment. Importantly, the assay should be done within the first 12 months after the reaction to reduce false-negative results (31-34).

The immediate allergic reactions to amide local anesthetics are considered very rare. However, the frequency of allergic reactions to them has recently increased, probably due to the preferential use of these anesthetics. The formulation of propofol (general anesthetic) contains other ingredients, such as refined soybean oil, medium chain triacylglycerols, injectable egg phospholipids, glycerol, and sodium oleate. Drug hypersensitivity in *in vitro* test might be to propofol or to one of the other components of the composition of the commercial preparation (35, 36). Zuo *et al.* (37) investigated the risk of true allergy to local anesthetics, they introduced only 6 from 68 patients with a suspected DHRs to local anesthetics and demonstrated positive results in skin tests and/or BAT. The authors conclude that skin tests and BAT may be reliable methods for investigation and diagnosis of true allergies to local anesthetics in clinical practice.

Anticoagulants belong to the most widely used drugs, all of them may provoke hypersensitivity reactions based on various pathogenetic pathways, different clinical manifestations and degrees of severity. We found a high percentage of positive BAT with these

drugs. Hypersensitivity reactions were formerly attributed to preservatives and contaminants, such as proteins of animal origin. Low molecular weight heparins cause especially a delayed-type, non-IgE-mediated hypersensitivity response. Among various types of skin reactions, immediate hypersensitivity reactions may be caused by chondroitin sulphate of the incomplete heparin purification. Nadroparin calcium is a heterogeneous combination of sulphated polysaccharide glycosaminoglycan chains. Heparins may possibly bind to multiple cell surface proteins (38-40). Previous studies have shown that heparin promotes both *de-novo* generation and activation of T regulatory (Treg) cells, which do not suppress rather induce activation of basophils (41, 42). The experimental results indicated that heparin may potentiate CD63 expression, potentially influencing exosome functions. Heparin may increase white blood cell counts and can bind a variety of signaling molecules such as growth factors, cell surface proteins of pathogens and most notably, cell adhesion molecules. These signaling molecules are involved in cell communication, acting as ligands, receptors and second messengers. The noncovalent interactions of sulphated polysaccharides with proteins effect changes in protein conformation, facilitate protein-protein interaction, and sequester proteins at the cell surface. Long-term nadroparin calcium injections can cause localized and generalized cutaneous ADRs, an increasing number of cutaneous ADRs have been reported (43-47). It is speculated that the hypersensitivity to nadroparin calcium may be related to its special antigenic determinants. Ebo *et al*. (48) presented a case of combined immediate and delayed type of hypersensitivity reaction to nadroparin calcium in a patient tolerant for unfractionated heparins and dalteparin. Ibuprofen is a commonly used antipyretic and analgesic nonsteroidal anti-inflammatory drug (NSAID). The ingestion of lipid transfer proteins (plant-food allergens) alongside NSAIDs (commonly ibuprofen) can trigger anaphylaxis, urticaria, and angioedema. ADRs to NSAIDs can be caused by specific immunological mechanisms (allergic reactions) or by biochemical processes linked to arachidonic acid metabolism, *e.g*. nonallergic hypersensitivity or cross-intolerance reactions. The precise patient phenotyping and pharmacogenetics information will improve the understanding and management of DHRs (49, 50). BAT, based on the detection of CD63 upregulation induced by NSAIDs, has been described. Positive tests were more frequent among patients having a severe hypersensitivity (grade II) contrasting to patients suffering from NSAID hypersensitivity restricted to cutaneous reaction (grade I). BAT is useful for the *in vitro* diagnosis of NSAID hypersensitivity, providing good specificity and positive predictive value and diagnostic reliability in the assessment of NSAID intolerance (51, 52). Metamizole belongs to the category of non-opioid analgesics, and as for other NSAIDs such as acetylsalicylic acid, diclofenac, or ibuprofen, both isoforms of cyclooxygenase (COX-1 and COX-2) are inhibited. IgE-mediated allergic reactions are found in only a small proportion of patients suffering from early onset reactions (53, 54). BAT has a good correlation with skin test results to metamizole and may be a useful diagnostic tool in patients with severe immediate allergic reactions to pyrazolones (55).

Paracetamol allergy is not common, and many of the reactions are related to the pharmacological action of COX-1 inhibition. Selective and IgE-mediated hypersensitivity reactions are rare. Cross-intolerance to NSAIDs was observed among 25% of patients with paracetamol hypersensitivity (56, 57). Bergeret *et al*. (58) reported a rare case of immediate allergic hypersensitivity to paracetamol with positive intradermal test and BAT. The authors concluded that BAT correlated with the relevant clinical history and can be a reliable tool to identify the culprit drug. We pointed out that antibiotics dominated in children, younger adults and older adults, in old age a positive response to hypolipidemics prevailed. Our data contrast with Accarino *et al*. (59) who show that the number of reported allergies to antibiotics increases with age, and that older adults have an increased risk of negative effects associated with alternative antibiotic use. BAT is an advantage from a clinical point of view, especially for a geriatric patients, who are often affected by multiple diseases.

Statins exhibit pleiotropic effects potentially affecting several immune response properties including immune cell activation, migration, cytokine generation, immune metabolism, and survival. They are derived from two origins: biological – extracted from *Penicillium* and *Aspergillus* fungal metabolites (*e.g*. Simvastatin), and synthetic (*e.g*. Atorvastatin, Fluvastatin, Rosuvastatin), and may upregulate proinflammatory cytokine production (60, 61). Kolawole *et al*. (62) demonstrated that Fluvastatin modulate basophils degranulation *in vitro*. Simvastatin was found to modulate the secretion of exosome from various cell types, CD63 decreased in dose-dependent manner (63).

We recorded a very low amount of positivity for hypolipidemic drugs; however, a higher frequency of positive response was detected in old age group. The CD63 and CD81 markers have been identified as indicators of biological aging. Exosomes play a significant role in cellular communication and significantly impact the aging process. Apoptosis is involved in aging and age-related diseases. In addition to inhibiting proliferation, Simvastatin was also found to promote cell cycle arrest and induce apoptosis. Statins primed apoptosis through its intrinsic pathway involving the mitochondria, the apoptotic pathway was presumably regulated by altered prenylation of cell signal transduction such as Ras and RhoA. The selective analysis of apoptotic *versus* nonapoptotic cells proved that both the increased expression of the tetraspanin CD63 and the loss of CD62L were restricted to the apoptotic subpopulation (64-69). Our study confirms a decreasing trend with age in the frequency of BAT positivity. Elderly patients had 7.6% positive responses and significantly differed in CD63 expression from all other groups, showing reduced expression of the receptor. DHRs may be less frequent and/or less severe in elderly possibly due to the involution of the immune system typical of this period of life. Ven-

tura *et al.* (70) report a rate of DHRs in the elderly of 30%, and the much higher activity explain by the fact that many affected patients were not referred for examination by a specialist. Polypharmacy is more common in the geriatric population as they suffer from multiple co-morbidities. Bielen *et al.* (71) have demonstrated that drug hypersensitivity was most common in young women aged 18-29 years, and the incidence of suspected drug hypersensitivity lowered with increasing age.

We found no gender differences in CD63 expression, in agreement with Koumaki *et al.* (22). We established the highest frequency of BAT positivity in some groups classified according to the ICD, D89 (other disorders involving the immune mechanism, not elsewhere classified) and L50 (urticaria). Mechanisms triggering the pathogenesis of chronic spontaneous urticaria (CSU) have been identified as type I autoallergic (which is associated with IgE antibodies against autoantigens) and type IIb autoimmune (which is driven by autoantibodies to FceR1 and/or IgE). NSAIDs have been found to exacerbate urticaria (NSAID-exacerbated urticaria) in 10% to 30% of patients with CSU (72, 73). Netchiporouk *et al.* (74) show that positive CD63 BAT are common in autoimmune CSU and are associated with high disease activity, children with CSU showed significantly increased BAT values compared to healthy controls.

Some controversies about classification of DHRs persist due to overlapping reactions and the appearance of similar symptoms, which may be caused by quite different immune and even non-immune mechanisms. We observed no differences between clinical manifestations of drug allergies (*e.g.* cutaneous, respiratory, gastrointestinal, cardiovascular, hypotension, confusion) and the activation marker CD63 and/or frequency of BAT positivity (data not shown). ADRs represent a major impact on society, increase morbidity, mortality, and healthcare costs. Considerable progress has been made in understanding the mechanisms of DRs, the physicochemical properties of drugs, the identification of risk compounds, and the specific characteristics of patient populations (75).

In conclusion, BAT is useful tool for diagnosing DHRs, predicting and monitoring clinical responses to treatment. This *in vitro* flow cytometric method eliminates the need for *in vivo* procedures that can cause unpredictable and severe reactions. BAT is a rapid and safe diagnostic tool for patients with drug-induced anaphylaxis. Further research is needed to standardize drug allergy phenotypes, endotypes and biomarkers.

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Contributions

MH, MS: conceptualization. JS, NM, MS: formal analysis, investigation. MH, MS: methodology. NM, JS: resources. MH, MS: supervision. MH: writing – original draft. MH, MS, NM, JS: writing – review & editing.

Conflict of interests

The authors declare that they have no conflict of interests.

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