Supplementary material to:

Immunological methods for diagnosis and monitoring of exposure-related type I allergic disorders caused by industrial sensitizing agents (IMExAllergy)

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Includes

Abbreviations

sIgE specific IgE
HMW high molecular weight
LMW low molecular weight
OA occupational asthma
OR occupational rhinitis
RADS reactive airways dysfunction syndrome
RUDS reactive upper airways dysfunction syndrome
WRA work-related asthma

Supplementary Methods

General approach

To develop this new practical guideline, we first assembled a task force from European Respiratory Society (ERS) members, which was constituted of a group of clinicians, allergologists, toxicologists, epidemiologists and public health specialists. The first group meeting was held during the ERS congress, September 29, 2015, in Amsterdam; development of a draft concept was initiated, including questions to be addressed, expertise and methods needed to accomplish the goals. At an early stage, it was decided to include additional broad laboratory expertise, e.g. from the UK, Spain, Germany, Switzerland and the US. Further, missing expertise in dermatology and from the ETN (Ear, Nose, Throat; Otorhinolaryngology) was included from international EU Cost project members (DiMoPEx) and the task force activities were included as a project in the DiMoPEx actions. Finally, four working groups were established, i.e. Clinical phenotyping, specific IgE (sIgE) testing, Exposure assessment, Integrated diagnostic approach.

First, it was decided that a systematic review and meta-analysis of the literature on existing sIgE methods and data will be performed. In other parts of the guideline illustrating the current state of the art knowledge along with appropriate examples was chosen by expert subgroups to illustrate the available methods, experience and their functional as well as medical significance.

The DiMoPEx has organized 3 day training school in Berlin at Charité Comprehensive Allergy Center, University Medicine, March 27 – April 1, 2018. This training school included representative presentations of available evidence followed by a Task force meeting, which reevaluated the aims and goals of the guideline in more detail, as well as current subgroup section work.

Second, the task force agreed on the following pertinent questions to address in this document:

- What are the clinical pictures of inhalant allergies?
- What are the test performances for high molecular weight allergens, notably of specific IgE?
- What are the test performances for low molecular weight allergens, notably of specific IgE?
- What is the test performance for recombinant or highly purified allergens?
- What are requirements for sIgE measurement?
- What is commercially available and suitable?
- How to standardize in-house testing and importance of quality control
- How are standardized in-house (dedicated) tests performed?

- What are the differences between the tests with HMW and those with LMW?
- Exposure assessment (sampling of air probes, dust and other material; role of safety data sheets (SDS); How do useful questionnaires look like?)
- How have samples of air probes, dust and other materials to be taken, handled and proceeded?
- Which are the needed/important and diagnostic methods for respiratory allergies?
- What is the best integrated diagnostic approach

So, four approaches were applied to perform these practical statements:

1. For the central chapter *Diagnostic measurement of specific IgE as the major step in identifying the precise cause of respiratory sensitization"*, a systematic review with meta analysis was performed, for details see below
2. For chapters *Clinical pictures*, *Allergen exposure assessment* and *Integrated diagnostic approach* the aforementioned working groups checked existing systematic reviews, meta analyses and statements of leading organizations relevant for this topic with special regard to the quality of the aggregated evidence available and prepared draft chapters with initial statements and recommendations.
3. Delphi conferences, telephone conferences and Skype meetings were held to reach consensus on the aforementioned draft with input from all task force members.
4. All statements and recommendations were based on above-mentioned approaches.

The scope of this guideline focuses on in vitro diagnostic methods for diagnosis of type I allergic disorders due to industrial agents. It also covers the role of this method in the overall diagnostic approach including exposure assessment. This includes current evidence on specific IgE testing in the diagnostics of allergic work-related asthma (WRA).

Definition of industrial sensitizing agents:
Allergenic substances found in workplaces or non-occupational settings where industrial (or agricultural) products are used or occur in airborne dust containing such product-borne sensitizers. Note that such sensitizing agents, present in the environment may differ from the occupational or food allergens originated from the same product (i.e. different soy allergens are known to be responsible for occupational asthma, for food allergy or allergic reaction from environmental dust exposure).

Systematic review and meta-analysis
According to the relevant questions identified, a MEDLINE search from 1992 till December 2016 was performed at the beginning. The current research was augmented by searches of EMBASE and TF member files.

The literature search was limited to manuscripts published in the English language and English abstracts available from articles published in other languages.

In order to provide extensive data on sIgE testing for occupational asthma, Harald Lux et al. conducted a separate systematic review with meta-analysis. Diagnostic power for high and low molecular weight allergens, allergenic components and the extent of standardization were the main objectives. Articles derived by an electronic search in EMBASE and MEDLINE, reference lists of detected systematic reviews and authors' collections of the period from 1967, the year of introduction of the first sIgE test (3), to July 2016 were the data basis of this analysis (4).

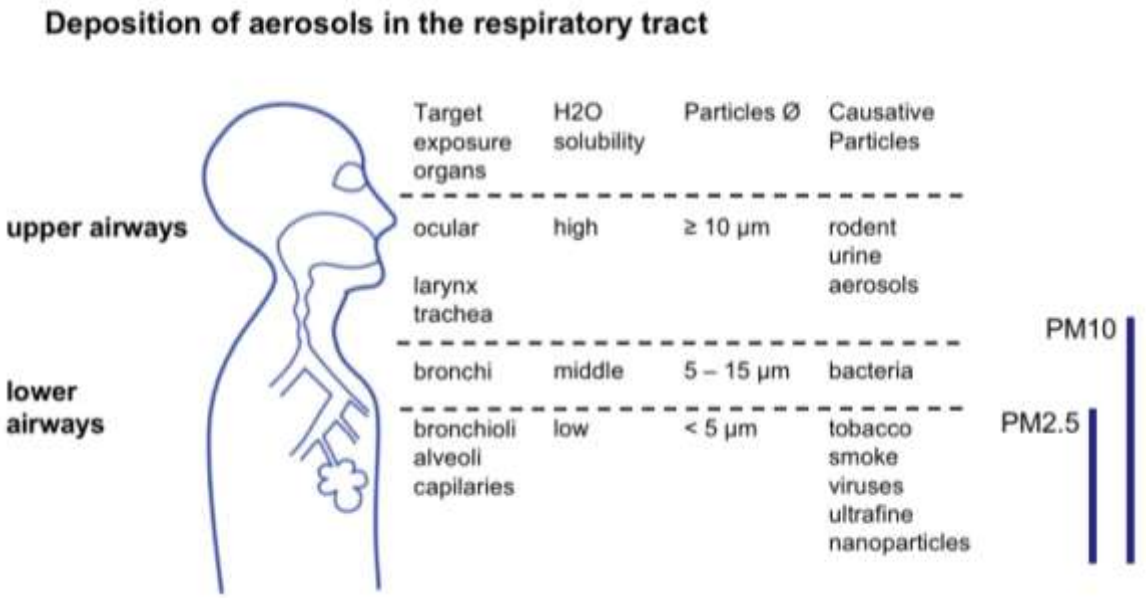
Supplementary material to: Clinical pictures of type I allergies caused by industrial sensitizers

a) Definition of the various diseases

Occupational asthma

Occupational asthma (OA) has become one of the most common forms of occupational lung disease in many industrialized countries having been implicated in 9-15% of adult asthma and work-related asthma is even more common (5) (6). OA is a disease characterized by variable airflow limitation and/or airway hyper responsiveness due to causes and conditions attributable to a particular occupational environment and not to stimuli encountered outside the workplace (7).

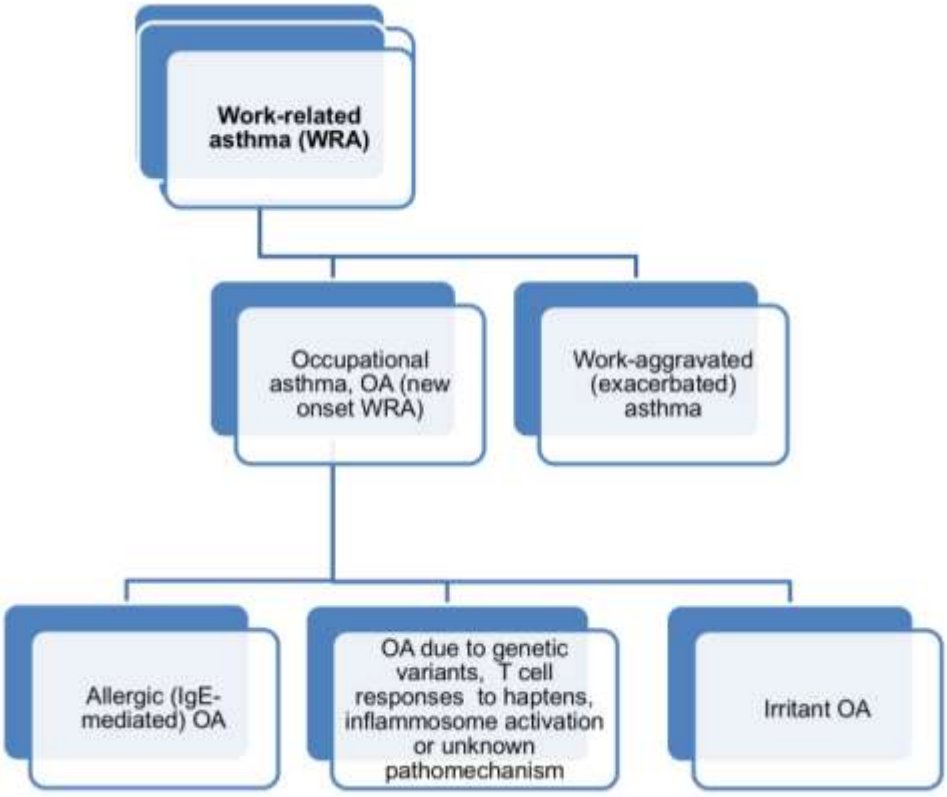
Supplementary Fig. 1: Deposition of aerosols in the respiratory tract



PM10 particulate matter: particles smaller than 10 µm;
PM2.5 particulate matter: particles smaller than 2.5 µm

Two main types of OA and several subtypes (some with hypothetical background so far) are distinguished by whether they appear after a latency period (see Supplementary Figure 2).

Supplementary Fig. 2: Subgroups of work-related asthma (WRA)



OA can be:

- Allergic (immunological): the onset of symptoms is after a latency period of exposure during which the worker becomes immunologically sensitized to the causal agent. This type encompasses OA that is induced by an IgE mechanism (most high- and a few low-molecular-weight agents), and OA in which an IgE mechanism has not been demonstrated consistently (low molecular-weight agents such as diisocyanates, western red cedar, and acrylates). It represents the majority of OA.
- irritant*: it is generally characterized by the absence of a latency period and occurs after accidental exposure to various concentrations of a workplace irritant (8, 9) This clinical entity has been defined as irritant-induced asthma and is less frequent than immunologic OA. The form of irritant-induced asthma occurring after a single exposure to high levels of an irritating, gas, vapor or aerosol was initially defined as “reactive airways dysfunction syndrome” (RADS) since there are some differences compared with typical asthma (10). However, subsequent long term follow-up of RADS demonstrated pathological features

consistent with asthma, such as thickening of subepithelial collagen and sub mucosal eosinophilia (11).There is a further subgroup where a pathological mechanism has not yet been clearly defined.

- There is evidence for rare additional subgroups such as specific genetic variants (12), inflammasome activation (e.g. by diesel exhaust) including non-eosinophilic responses (13),T cell responses to haptens; furthermore, there remains a group with unknown pathomechanisms

*Irritant-induced asthma: An issue is whether asthma develops from multiple intermittent exposures to high levels of irritants, leading to progressive changes not measured before clinical onset. It is more controversial whether chronic low-level exposure to irritants can cause OA. A position paper of EAACI concluded that the causal relationship between irritant exposure(s) and the development of asthma can be substantiated by the temporal association between the onset of asthma symptoms and a single or multiple high-level exposure(s) to irritants, whereas this relationship can only be inferred from epidemiological data for workers chronically exposed to moderate levels of irritants. Accordingly, the following clinical phenotypes should be distinguished within the wide spectrum of irritant-related asthma (IIA): (i) definite IIA, that is acute-onset IIA characterized by the rapid onset of asthma within a few hours after a single exposure to very high levels of irritant substances; (ii) probable IIA, that is asthma that develops in workers with multiple symptomatic high-level exposures to irritants; and (iii) possible IIA, that is asthma occurring with a delayed-onset after chronic exposure to moderate levels of irritants (14).

Work-aggravated asthma

In the broad group of work-related asthma, work-aggravated asthma should be included, and is defined as preexisting or concurrent asthma that is exacerbated by workplace exposure (9, 15) (Supplementary Figure 3). Exercise and exposure to cold dry air, dust, fumes, and sprays are common in the workplace and may aggravate asthma especially in those with moderate to severe disease and in those not receiving optimal treatment. Work-aggravated asthma should be distinguished from OA, because the origin, outcome, medical management, and preventive measures differ substantially. Reducing workplace exposure to respiratory irritants, limiting exposure to relevant environmental allergens and non-occupational irritants such as tobacco smoke, and optimizing anti-asthma therapy often allow workers with this type of asthma to be retained in the same job. Some individuals with work-aggravated asthma, as a consequence of new exposures to specific agents in the workplace, may develop true OA. In any case of work-related respiratory symptoms, the diagnostic process to exclude true OA should be performed.

Variants of occupational asthma

Work-related asthma encompasses variant syndromes including eosinophilic bronchitis and asthma-like disorders caused by exposure to organic dusts.

Eosinophilic bronchitis*:* Eosinophilic bronchitis has been described as a cause of chronic cough characterized by sputum eosinophilia in the absence of demonstrable variable airflow limitation

or non-specific airway hyper-responsiveness (16). Exposure to acrylates, natural rubber latex, mushroom spores, and an epoxy resin hardener in the workplace can result in increased sputum eosinophilia in the absence of airflow limitation and increased non-specific airway responsiveness (17). Since increases in sputum eosinophils are remarkable and reproducible, the condition should be considered occupationally-induced. Although this condition clearly does not meet the definition of asthma, it is not known whether it can progress to typical OA.

Asthma-like disorders

Asthma-like disorders have been especially related to exposure to organic dusts such as grain, cotton, and other textile fibers and to dust from animal confinement buildings. These disorders are different from OA since respiratory symptoms may be associated with systemic symptoms, there is no latency and the onset can occur in naïve subjects on first exposure. Asthma-like disorders may be associated with increases in non-specific airway hyper responsiveness, but milder and transient compared with OA. Finally, in asthma-like disorders, asthma symptoms may be associated with neutrophilic airway inflammation, which has been shown for few causal agents of OA (18). Asthma-like disorders have been associated with exposure to endotoxin, and this observation makes their mechanism more intriguing.

Occupational rhinitis

Occupational rhinitis (OR) is defined as an inflammatory disease of the nasal mucosa, characterized by intermittent or persistent symptoms (i.e., nasal congestion, sneezing, rhinorrhea, itching, and/or hyper secretion) due to causes and conditions attributable to a particular work environment and not to stimuli encountered outside the workplace (1), .

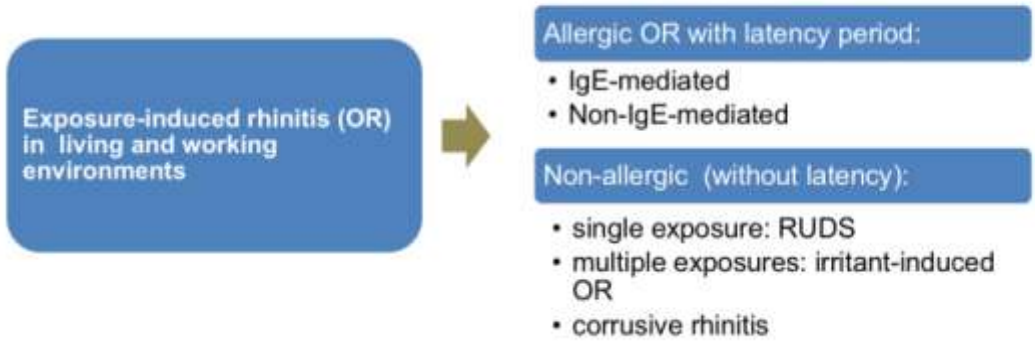
The majority of patients diagnosed with OA also suffer from OR (up to 92 % of cases), which most often precedes the development of OA. Allergic OR due to high molecular weight agents tends to be 3 times more prevalent than allergic OA. Recognition of OR is important for the prevention of occupational asthma (19).

Different forms of OR have been identified and the classification of OR resembles that of occupational asthma (Supplementary Figure 3)

Allergic OR is characterized clinically by the development of nasal hypersensitivity to a specific occupational agent appearing after a latency period, which is necessary to acquire immunological sensitization to the causal agent mediated hypersensitivity reactions (which is resulting from antibody- or cell-mediated mechanisms). The symptoms can be intermittent or persistent depending on the frequency and intensity of exposure to the causal agent. OR encompasses both allergic IgE-mediated OR and non-IgE-mediated OR.

Non-allergic OR includes different types of rhinitis caused by the work environment through irritant, non-immunological mechanisms. Acute onset *'irritant-induced OR'* usually occurs without a latency period. This entity is quite similar to the situation of RADS so that the term '*reactive upper airways dysfunction syndrome*' (RUDS) has been proposed.

Supplementary Fig. 3: Subgroups of rhinitis (from (1))



The term '*irritant-induced* OR' may also refer to symptoms of rhinitis reported by subjects repeatedly exposed at work to irritants without identifiable exposure to high concentration of irritants.

The term 'corrosive rhinitis' has been used to describe the most severe form of 'irritant-induced OR', which is characterized by permanent inflammation of the nasal mucosa (sometimes associated with ulcerations and perforation of the nasal septum) that may develop after exposure to high concentrations of irritating and soluble chemicals.

A pre-existing or concurrent (allergic or non-allergic) rhinitis, not caused by a specific agent in the work environment, but that is worsened by workplace exposures is defined as work-exacerbated rhinitis (19).

Ca 70% of patients with OR exhibit non-specific nasal hyper responsiveness. Non-specific nasal hyper- responsiveness is defined as one or more nasal symptoms (sneezing, rhinitis or nasal obstruction) following the environmental stimuli cigarette smoke, temperature or humidity changes, strong odors/fragrances, and other irritants (20).

Contact urticaria and protein contact dermatitis

This disorder is caused by contact with proteinaceous material and some low molecular weight chemicals such as organic acid anhydrides, diisocyanates, aldehydes; it mostly occurs in occupational settings (21, 22). It is characterized by an acute urticarial or vesicular eruption occurring minutes after contact with the causative protein. Immediate prick- or scratch-test results are usually positive thus confirming an immediate type allergic reaction. These agents usually cause dermal reactions at the site of direct skin contact (mainly the hands), but airborne

dermatitis with facial involvement can also occur. [Of note, chemicals that cause allergic contact dermatitis by non-IgE mechanisms, such as metals (Cr, Ni, Co) or synthetic agents (pharmaceuticals, reactive chemicals), can also cause airborne dermatitis and occupational asthma; in some patients, both dermal and mucosal reactions can be observed]

The following groups of proteins causing protein contact dermatitis can be differentiated:

- fruits, vegetables, spices, and plants; they cause the disorder mostly in kitchen workers, caterers, food vendors, food packers, and gardeners; further causative agents of this group comprise apples, asparagus, banana, bean, carrot, chrysanthemum, cornstarch, mugwort, natural rubber latex, paprika, peach, peanut, pear, shiitake mushroom, soy;
- flour, predominantly rye and wheat, which can also cause generalized dermatitis involving the face;
- proteolytic and other enzymes, which are relevant especially in the manufacture of detergents, **bakers**, pharmaceutical workers, and chemical enzyme factory workers. For the dominating respiratory symptoms caused by such enzymes see (23);
- animal proteins eliciting the disorder in **slaughterhouse workers**, **butchers,** commercial anglers, cooks, farmers, and **veterinarian**s; those in contact with animal intestines are most susceptible; here, common triggers include blood, bovine amniotic fluid, cheese, cow dander, egg yolk, maggots, meat, milk, salmon, squid, worms.

b) Example of allergic asthma due to environmental exposure to agricultural allergens not identified for more than a decade

One of the prominent type I allergies induced by the exposure to industrial raw products not causally identified for more than a decade are asthma epidemics in the harbor cities of Barcelona, Cartagena, La Coruna, Naples, New Orleans etc. They were shown to be due to allergens in soybean dust polluting the cities in wider areas outside the unloading places. In Barcelona, between 1982-1987, 26 single epidemics occurred with 1,155 hospital admissions and 20 fatal cases, described for the first time in 1989 by Sunyer et al. (24) identifying soy allergens as the cause for epidemics. Unlike the known soy bean caused food allergies or occupational asthma caused by exposure to soy in workplaces, different allergens present in dust from soy hull that polluted the city areas from unloading activities in the harbor were responsible for the pandemic type I allergy (25). Further purification and characterization of the allergens Glym IA and Glym IB identified the underlying specific IgE-mediated sensitizations (26). After 1994/1996 new cases of asthma occurred, precaution monitoring of the new recognized soy aeroallergens was initiated (27, 28).

Supplementary material to: Causal agents

Supplementary Table 1: List of Allergens causing occupational asthma by evidence. (modified from (29)

Strength of evidence for occupational asthma-caused by allergens according to the modified RCGP three-star system Evidence level (modified RCGP three star grading)	Number of allergens	Individual allergens
***	1	Co-exposure to various lab animals
**	18	Alpha-amylase from *Aspergillus oryzae*; various enzymes from *Bacillus subtilis;* papain; bakery (flour; amylase; storage mites); western red cedar; latex; *Psyllium*; farming (animals, cereal, hay, straw and storage mites); storage mites; rats; carmine; egg proteins; Atlantic salmon; fishmeal; Norway lobster; prawns; snow crabs; seafood; trout and turbot; reactive dyes; toluene diisocyanates (TDI); platinum salts;
*[*]	17	Detergent enzymes, soybean (husks, flour); paprika; tea dust; tobacco; *Aspergillus niger*; cows; predatory mites; spider mites; opiates; methylene diphenyl diisocyanate (MDI), phthalic anhydrides; various isocyanates
*	18	Eastern white cedar; various flowers; guar gum; poppy; rose (*Rosa rugosa*); senna; ispaghula husks; sunflower pollen; trypsin; various woods (abies, chestnut, douglas, framire, mansonia, oak, obeche, walnut, white poplar); weeping fig; nonbiting midges; hexahydrophthalic anhydride; hexamethylene diisocyanate (HDI); methyl tetrahydrophthalic anhydride (MTHPA); persulfate salts; polyfunctional aziridine
[*]	24	Alternaria; bromelain from *Ananas comosus;* cellulase from *Trichoderma viride*; lactase from aspergillus; various enzymes; chrysanthemums; castor beans; madagascar jasmine; pine; flowers; budgerigar; flour moth; house dust mites; mouse; poultry; red soft corals; screw-worm fly; shrimp; various birds; cephalosporine; penicillins; phenylglycine; acid chloride; trimellitic anhydride
(*)	19	Aspergillus enzymes; cellulase from *Trichoderma reesei;* pancreatin; proteolytic enzymes; asparagus; *Boletus edulis*; carnation; garlic dust; rye flour; gum arabic; iroko; various woods; African maple; black bat; mealworm; poultry mites; tetrachlorophtalic anhydride; chloramine T; chromium and nickel
-	275	Unlisted as no corroborating scientific evidence presented

Supplementary material to: diagnostic measurement of specific IgE as the major step in identifying the precise cause of respiratory sensitization

a) In vivo test: skin testing

Allergy skin testing was used for the first time in 1865 by Blackley (scarification) and later, in 1970, the intraepidermal technique (skin prick testing, SPT) with the modifications made by Pepys (30) was definitively introduced as a diagnostic method.

The methodology used in skin prick testing with industrial sensitizing agents does not differ from that used for common aeroallergens (pollens, mites, etc.) (31).

Skin prick testing of immediate hypersensitivity with industrial sensitizing agents is intended to demonstrate patient sensitization to a particular agent. Sometimes, commercial allergen extracts are available and standardized. These extracts, generally aqueous, are prepared in 50/50% saline/glycerol solution. Glycerol, in addition to providing greater stability, makes the solution more viscous, allowing the drops to stay better on the patient's skin.

The skin prick testing consists of placing a drop of the allergenic extract on the skin of the patient to be evaluated, and is punctured with a lancet (30) (Supplementary Figure 5b). It is estimated that with this method about 3 nL of the extract is introduced into the skin (30). Alternatively, a drop can be applied onto a previously made superficial scratch in the epidermis (scratch test). Positive (histamine) and negative (vehicle) controls are required. The reading is made at 15-20 min. The standard and accepted method for quantifying the skin prick reaction is to measure the mean diameter of the wheal, using a ruler marked in mm (a transparent ruler is often most convenient; calipers are also available for this purpose). If the result is a circular wheal, one measurement of the diameter (in mm) is sufficient; if ovoid or irregular, it should be measured on the longest and shortest perpendicular axis and the numbers are added and divided by 2 (mean diameter). It is recommended to keep a copy of the wheal in the patients records, for later evaluation. The wheal outline is drawn outside the perimeter, and the re-reading will be the diameter of the polygon inside the primeter-marking.

Intradermal skin testing can be performed when the skin prick testing is negative and the implication of the allergen is still suspected by the clinical history. The intradermal skin testing consists of an intradermal injection of 0.02 to 0.05 mL of the allergenic extract using an tuberculin syringe. It is basically identical to the one used for the Mantoux tuberculosis test. Although there are several methods of grading the response, they are usually expressed by measuring the wheal and flare reaction (surrounding erythema), and the occurrence of pruritus should also be taken into account.

Generally, in vivo test with HMW allergens using appropriate commercial extracts are positive when they are the cause of the disease and, thus, are useful in identifying the agent responsible.

When appropriate occupational sensitizing HMW antigens are tested, the sensitivity and specificity are similar to those found with the usual aeroallergens.

According to clinical experience intradermal skin testing is more sensitive and less specific than skin prick testing; its interpretation is more difficult and presents more false positives, thus resulting in a lower positive predictive value. With both tests, false positives and false negatives may occur due to bad technique. Even when the technique is correctly performed, false positives and negatives may occur due to use of an inappropriate material, because the extracts used are commonly not standardized and are of unknown composition, immunological activity or potency.

A major problem in skin testing is the need for standardized materials. Such materials are rarely available for cases with suspected occupational allergy. Hence, crude extracts produced locally have to be prepared with significant limitations in sensitivity and specificity. With the exception of platinum salts and reactive dyes, SPT is not reliable the diagnostic procedure in LMW allergy cases.

b) Early biomarkers of allergic sensitization, mediators and cellular tests used in diagnostics

Beside specific IgE, a classical biomarker of effect there are several other biomarker of early allergic sensitization described below.

Histamine release test:
The quantification of histamine release is a useful method in the in vitro study of allergic reactions. It allows evaluating the magnitude of the sensitization towards an antigen and to monitor this sensitization in the course of time (32). In general, there is a first phase of stimulation in which the cells of the patient are incubated with the different allergens to be tested. Subsequently, the released histamine is quantified for which we have different quantification methods: RIA, HPLC, ELISA, fluoro-immunoassay, etc. There are several commercial kits on the market to quantify histamine in fluids. The result is considered positive if there is an increase of histamine in the sample of more than 10% with respect to the control sample. The main problem of the technique is that the released histamine is at very low levels (in the order of picogram), so methods with minimal sensitivities of this order are necessary.

Tryptase:
Tryptase is a protease of human mast cells, considered a marker of mastocyte reactions, which is released after an inflammatory-type stimulus along with histamine. In a normal individual serum tryptase is undetectable. The determination of tryptase today is indicated in anaphylactic reactions (33), although some authors also use it in the monitoring of antigen-specific provocation tests. Tryptase is quantified in general by sandwich immunoassays. Two forms of tryptase, the so-called α and β-tryptase, have been described. The α-tryptase is the predominant form in the peripheral circulation both in basal conditions in normal subjects and in

patients with systemic mastocytosis, being very high in the latter. However, the form that rises predominantly when there is an allergic reaction is β-tryptase. Currently there are commercial methods and monoclonal antibodies for the standardization of the immunoassay in the laboratory.
Tryptase levels are elevated in anaphylactic reactions and systemic mastocytosis. Tryptase increases from 15-30 minutes after the onset of symptoms, reaches its maximum level from 60-120 minutes and returns to baseline values between 12-24 hours after the reaction (34) . The normal ranges are those specified by the manufacturer, in the case of using a commercial kit, or should be established by each laboratory in the case of using in-house developed tests.

Basophil and mast cell activation test:
The basophil activation test is based on measuring the percentage of basophils that are activated in contact with the allergen (35). Basophils carry antibodies of IgE type in their membrane. If the patient has specific IgE against an allergen, the basophils are activated upon contact with it. During activation, basophils express the CD63 molecule on the surface. The expression of this marker correlates with degranulation, which makes it a suitable marker to measure basophil activation. For the measurement, first, the leukocytes are separated by a gradient, in order to have a sufficient number of basophils. Allergen dilutions are prepared and concentrated leukocytes are incubated with the dilutions of the allergens. Subsequently, the expression of the CD63 marker is measured by flow cytometry using monoclonal antibodies. The activated basophils will express more CD63 marker on their surface. It is necessary to establish in each laboratory the cut points of the percentage of activated basophils that is considered clinically relevant.

Similarly, in the mast cell activation test (MAT) human blood-derived mast cells are generated from peripheral blood and incubated with allergens; degranulation is measured by use of flow cytometry and mediator release. Recently, Bahri et al. compared MATs with existing diagnostic tools in a cohort of peanut-sensitized subjects undergoing double-blind, placebo-controlled challenge. Human blood-derived mast cells sensitized with sera from patients with peanut, grass pollen, and wasp venom allergy demonstrated allergen-specific and dose-dependent degranulation, as determined by expression of surface activation markers (CD63 and CD107a) and functional assays (prostaglandin D2 and beta-hexosaminidase release). MAT was found to have superior discrimination performance compared with other testing modalities, including component-resolved diagnostics and basophil activation tests. The authors of this publication concluded that MAT is a robust tool that can confer superior diagnostic performance compared with existing allergy diagnostics (36).

Eosinophil cationic protein:
Eosinophil cationic protein (ECP) is a protein found in the granules of eosinophils (37). Patients suffering from allergic processes such as bronchial asthma trigger in their inflammatory process the appearance of ECP, which is released from eosinophils in the serum, bronchoalveolar fluid, nasal secretions and sputum (38). The determination of ECP can be an indicator of the degree of

inflammation as well as an indicator of the efficacy of the anti-inflammatory therapy established. ECP can be measured in both serum and plasma. However, ECP levels are much higher and consistent in the serum. Blood samples should be taken in tubes with separating gel and the coagulation will be carried out for one hour at 22 ° C before centrifugation and subsequent separation of the serum. Both glass and plastic tubes can be used, however the differences in the materials to be used and the inclusion of the coagulation activators in the tubes can affect the ECP levels (39). ECP can be quantified by specific immunoassays, and there are different alternatives in terms of commercial methods. Due to the importance of correctly obtaining the sample, the normal ranges for measuring the protein have to be established according to the conditions of each laboratory and not specifically following the manufacturer's recommendations. The increase in ECP is well related to the increase in eosinophils; on the other hand, the decrease of the ECP is related to the reduction of asthma attacks and the improvement of lung function. It is a marker of inflammation that helps us evaluate the effectiveness of the treatment; however, the elevation of the ECP can also occur in other pathologies involving the activation of eosinophils, such as atopic dermatitis, parasitic diseases, etc.

c) Measurement of specific IgE in serum

The principle of the in-house CAP assay is a **fluorescence enzyme labelled immunoassay** using the non-commercial CAPs coupling system (provided by Phadia/Fisher scientific). The protein is biotinylated since the alpha amino acid group on the N-terminus of the allergen reacts with biotin-XX-NHS (D-biotinyl-ε amino carbonic acid-n-hydroxyl succinimidyl ester) forming a stable amide binding (23). The molecular weight of the protein needs to be established prior to biotinylation using SDS-PAGE where the protein is calibrated against a standard curve of known molecular weight (MW) markers. The biotinylated protein is bound to commercial Streptavidin-CAPs (provided from Phadia) creating one of the strongest non-covalent bonds. In house coupled CAPs can be used in the same semi-automatic system as those of commercial CAPs, where specific IgE is expressed in kilo units per litre (kU/L)) and corrected with the WHO reference of human serum IgE; 1 kU=2.4 ng/L (23). Note, that in-house RAST assays can be superior to commercial CAP assays, e.g. with acid anhydrides and isocyanates (40); for more details see Supplementary Table 3 below.

Sometimes crude extracts from different allergen sources are used for detection of specific IgE. The composition and amount of allergenic extract strongly affects the results. Often crude allergen extracts are run on a separation gel followed by blotting with serum containing specific IgE to help identify the allergen. This method known as immunoblot analyses is commonly used for workplace specific proteins (4, 40-46) . For this in-house immunoblot analysis, the samples are mostly lyophilised, reconstituted in 10% of the original volume, and then stored at -20°C (note the protein content has to be determined and additionally controlled with non-lyophilized extracts in SDS-PAGE analysis) (47, 48). The detection of the allergenic proteins in the extracts is performed by immunoblotting. Proteins are separated by SDS-PAGE (mostly 15% gels to achieve optimum separation of proteins in the relative molecular mass between 6 kDa and about 60 kDa. Molecular weights estimated by comparison to commercial MW standard mixtures).

Immunoblot experiments are performed with specific proteins, for every patient separately, with extracts from the lyophilised raw material specific for working environments. Equal amounts of the proteins are separated on SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes in a semi-dry blot. The membranes are treated using standard laboratory western-blot procedures (incubated with anti-human-IgE monoclonal antibodies. Specific protein bands are detected with either phosphatase or peroxidase-based methods) (46).

Identification of soy hull allergen was verified by immunoblot: soy aeroallergen environmental exposures might vary markedly between worksites where soy is handled. Two LMW proteins Gly m 1 and Gly m 2 have been identified as the main allergens that cause such asthma attacks (49). Rodrigo et al. (25) demonstrated by Western blot that patients with asthma during an asthma attacks reacted strongly to glycoprotein bands with molecular weight less than 14.4 kd, and isoelectric point less than 6, which appeared to correspond with Gly m 1 protein. Later on, Codina et al. (49) demonstrated the existence of another soybean hull allergen, Gly m 2, partially responsible for the soybean asthma outbreaks. So, for immunoblot analysis of patient's IgE antibodies it is necessary to prepare a soy hull LMW Extract (SHLMWE). A valid method to analyze the IgE antibodies with the help of SHLMWE is a chromatographic process (using CMcellulose and DEAE-cellulose chromatography) (27).

In-house ELISA (Enzyme-linked Immunosorbent Assay) assays are based on a method attaching antigens to a surface coupled with antibody (usually a polystyrene microtiter plate, mostly with 96 wells). A specific secondary antibody has to be applied over the surface and can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate (which is a phosphatase or peroxidase). The subsequent reaction produces a detectable signal, most commonly a color change in the substrate (detected by ELISA reader). Performing an ELISA involves at least one antibody with specificity for a particular antigen, meaning that a quality of a specific primary antibody to the protein which is intended to be analyzed, is a rate limiting step in any ELISA assays (especially for in-house ELSAs. Note that it is also applied to commercially available assays). Any in-house ELISA needs to provide detailed information on the antibody/antibodies used.

In-house sIgE tests

Immunoassay with non-commercial CAPs: Coupling to unknown protein: The principle of the method: free amino acid group of the marked protein will react with biotin-XX-NHS (D-biotinyl-(amino carbonic acid-n-hydroxyl succinimidyl ester, i.e. from Sigma) forming a stable amid binding. Non-reacting biotin-XX-NHS is separated on Sephadex G25 column. The biotinylated protein can be bound to commercial Streptavidin-CAPs (o212, Streptavidin CAPs, provided from Phadia) creating one of the strongest non-covalent biological binding. Individual CAPs created in that way can be applied analog to the known commercial immunofluorescence method (i.e. CAP-Phadia/Fisher) from described below. If the molecular weight (MW) of the analyzed protein is unknown (in this case the protein is a work place specific allergen), the MW has to be determined in a separate step; using standard procedure a SDS-PAGE is performed and the

MW of the protein is calculated with a help of calibrated commercial MW- marker (standard curve).

The creation of non-commercial CAPs follows the established (23) in-lab standard protocol: 3 mL allergen solution (i.e. workplace specific enzyme proteins) using biotin-XX-NHS in the ratio 5:1 (5 mole activated biotin to 1 mol protein) biotinylated for 2h. For the calculation the following equation is used: [(protein concentration in mg/mL) x (volume of the protein solution in mL)/ estimated (see above) or known MW of the analyzed protein in Dalton)]x5x567,7 (mg/mmol)/20 mg/mL) x1000 (µL/mL) volume of biotin-XX-NHS in µL. The purification of the mixture takes place over Sephadex G25 column, pre-washed with 1% BSA solution and 1xPBS solution. The column is eluted with PBS and 9 fractions are collected. The protein concentrations from each fraction should be measured separately (i.e. using Bradford assay method from BioRad, or other provider). The first 3 protein collecting samples are merged together. If the protein concentration exceeds the concentration of 1.4 mg/mL, the sample has to be diluted and repeatedly measured. The determination of the biotinyliation grads is carried out by a photometric measurement of the ΔOD500 / HABA / avidin / sample over a HABA / avidin kit (i.e. from Sigma). To calculate the biotinylation rate the following equation is used: botinylation rate=dilution factorx10x0.9x (OD500/HABA/Avidin)- OD500/HABA/Avidin/sample(]/34 [mean protein concentration from 3 column fractions in mg/mL) (MW of the protein to be analyzed)]/1000.

Specific IgE measurements with LMW agents such as isocyanates (MDI) by ImmunoCAP (40)

LMW allergens are too small to be recognized by the immune system without conjugation to a protein (see supplementary table 2 for details on conjugation).

The preparation of MDI-HSA conjugates in-vapor and in-solution has been published (in (50) (51) (52-54)). The in-vapor method is based on a specially constructed 2 chamber-system used to fumigate the human albumin (i.e. 99 % pure, globulin free, Sigma, Germany) solution with vaporized 4,4 MDI (pure analytical standard, i.e. Riedel-de-Ha¨en, Sigma, Germany). Individual conjugates, were coupled with biotin and used for the fluorescence enzyme immune assay detection method (i.e. semi-automatic ImmunoCAP100, Phadia, Freiburg, Germany). Serum-specific IgE is expressed in kilo unit per liter (kU/L) correlated with the WHO reference of human serum IgE (1 kU = 2.4 ng/mL). A dose–response calibration (i.e. seven points) should be performed for each IgE and IgG measurement. The limit of detection (LOD) for specific IgE is 0.02 kU/L, 0.2 mg/L for IgG and 100 kU/L as the limit of calibration similar to that used for commercial Immunofluorescence assays. The following controls were included for the validation of the assay: pooled positive and negative patient/control sera, analytical standards (also used as set points for quality control), HSA solution and biotin control samples. The measured day to day precision was <12 % RSD. The assay validation was performed according to the good laboratory practice. Separate studies with HSA solution showed that IgE values above 0.02 kU/Land IgG values above 3 mg/L can be considered as specific (above means + 2 RSD or 10 % analytical variation). The variability between the in- vapor method and the commercial assay

method was: 0.5–20 % (for lower and upper edge of failure) for the IgE values. For the IgG data, however, the values collected with commercial CAPs were continuously 5–35 % higher in all tested subjects.

Detection of MDI-bound to HSA:

Example from Budnik et al. (40) describe the evaluation of the in-house methods: The MDI-HAS conjugates were subjected to SDS-PAGE using a 9 % separation gel. The amount of MDI-bound to HSA was calculated from the intact protein shift using MALDI-TOF-MS (using CHCA- matrix) and compared with non-conjugated HSA. Immunological data were expressed as mean value. By applying this in-house method (40) each analysis was repeated at least twice with three independent preparations (except for the assay validation). For the comparison of the binding data between the sera for variously responding patients, the data for each individual patient were transformed into a percentage of maximal binding. The patient sera were measured first individually, and then the samples were pooled as follows: all IgE-positives (median, 26 kU/L) gave one pool, all IgG-positives (median, 13 mg/L) gave another, and two control pools (healthy group and baker' asthma patients) were the third and the last group. To test individual conjugates and to validate the assay, a pool serum from isocyanate asthmatics was used. All immunological methods were validated routinely with control serum samples and additional standard set points (two analytic standards, one low and one high concentration were used as set points).

Supplementary table 2: *Conjugate preparation for in vitro specific IgE testing*

Conjugate preparation for in vitro specific IgE testing Low molecular weight allergens need to conjugate to a protein to be recognized by the immune system
Recent studies highlighted the difference in specific IgE binding according to the conjugate preparation conditions (43) (40). One of the difficulties with measuring specific IgE to low molecular weight allergen is that there are no standardised protocols for the preparation of protein-LMW allergen conjugates and characterization of the resultant protein hapten complex can be technically challenging and complex. There were various approaches to bind LMW agents such as acid anhydrides (55, 56), glutaraldehyde (57) or isocyanates (for details by use in vapor and in solution approaches see example with MDI and acid anhydrides in Table 3 of the Supplementary material) to proteins in order to detect specific IgE antibodies. The diisocyanates are emblematic of difficulties of elucidating underlying antigenic forms of diisocyanate haptenated proteins. Albumin is the preferred protein carrier, but multiple proteins are potentially haptenated in vivo (see Figure 2, main manuscript body). Diisocyanates have been reported to covalently bind to albumin, haemoglobin, lung epithelial proteins, tubulin and keratins (58, 59),(60, 61)). Proteomic mass spectrometric studies of in vitro conjugation of diisocyanate to albumin and haemoglobin has demonstrated that diisocyanates can form a variety of complexes with proteins including inter- and intramolecular cross linked species (see Figure 3, main manuscript body); one diisocyanate binding through a urea linkage with the other hydrolysizing to a free amine; and self-polymerization of multiple diisocyanates onto that

free amine formed. Electrophilic LMW allergens react with protein nucleophiles (thiols, N-terminal α-NH2 and lysine residue ε-NH2s.) Multiple concentration dependent diisocyanate bindings sites on albumin and haemoglobin have been have been identified (62, 63). Similarily hexahydrophthalic anhydride has been shown to bind to multiple sites on albumin (64). Buffer system and pH can also influence haptenation. While in general, the higher conjugation reaction ratios of hapten to albumin provide better sensitivity in detection of specific antibody (antigenicity), it is not known if that is due to the increased total amount of the hapten bound or the formation of additional antigenic forms. The polyclonal nature of the antibody response and potential cross-reactivities makes this very difficult to tease out. Such complexity underlines the hurdle that must be overcome to develop consistent, well characterized LMW hapten-protein antigens for use in patient diagnosis.

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The WHO human IgE reference preparation

Specific IgE assays are standardized using a World Health Organisation reference preparation of human IgE. Until recently the second WHO international standard 75/502 was used, however as the stock of this is depleted, the third WHO international standard 11/234 human IgE reference preparation is being used (http://www.who.int/biologicals/BS_2220_Candidate_Preparation.pdf).

Hamilton (65) who has been much engaged in IgE standardization concludes: "*Convergence and harmonization of technical factors has led to improved agreement among reported IgE antibody results, specific IgE antibody levels, as measured with different commercial assays, are still not interchangeable or identical. Differences remain in the specificity of the allergen-containing reagents used in the different assays":*

Allergen preparation for use in specific IgE assays

There can be a significant degree of heterogeneity in the allergens used in specific IgE assays which can impact on the quality of the assay. Mostly allergens are mixtures of proteins prepared from biological extracts that differ in their composition between manufacturers, because of several factors:

- season when material collected
- Degree of difficulty in identifying a pure source of material
- Presence of morphologically similar raw materials that might cross- contaminate
- Differences in extraction and final processing during allergen reagent production by assay manufacturers
- Quality control:

It is important to establish the composition of the allergen by running the extracts on isoelectrofocusing, SDS-PAGE, crossed immunoelectrophoresis and immunoblotting methods.

Allergenic potency can be assessed by using a soluble antigen inhibition format of the allergen-specific IgE assay and the stability of the extracts needs to be established during storage. Heterogeneity of the human IgE antibody-containing sera can be used for quality control. Manufacturers are also likely to have different criteria for acceptance of the finished allergen-containing reagents. Allergen-containing reagents from different manufacturers are likely to detect different populations of IgE antibodies for any given allergen specificity.

Supplementary material to: Allergen Exposure assessment

a) Dermal exposure as causes of type 1 allergies

Several lines of evidence exist that suggest the skin may be an important route of allergic sensitization that can lead to respiratory hypersensitivity diseases (asthma) upon subsequent chemical allergen challenge to the respiratory track. The literature relevant to the potential importance of skin exposure in the development of asthma has been the subject of several reviews (59, 66-68). Animal studies have demonstrated respiratory hypersensitivity and inflammation upon organic acid anhydrides and diisocyanates specific respiratory tract challenge in dermally sensitized rodents. Dermal chemical hapten exposure, not only stimulates local lymph node cells, but those peripheral to the site of exposure suggesting that dermal exposure can produce systemic chemical allergic sensitization (69). Evidence that dermal sensitizing exposures lead to asthma in humans is limited and largely based on circumstantial evidence. These include case reports, epidemiologic studies reporting increased risk of isocyanate asthma with dermal exposure, and in workplaces with isocyanate asthma demonstration of dermal isocyanate exposure in settings with very low to non-detectable air levels. It is also noteworthy that 7 of the 10 most common occupational contact allergens are possible causes of occupational asthma (70). It has also been proposed that those with an impaired skin barrier may also be more susceptible to systemic sensitization by proteins entering through the skin and this can lead to other immune hypersensitivity diseases, including asthma. This concept has been termed “the atopic march” (71).

b) Air sampling methods and databases

The majority of aerosols are collected by filtration on a membrane available in various pore sizes and materials (Teflon ®, cellulose esters, polyvinyl chloride, glass fibers, silver, or other materials) in a plastic cassette placed in the breathing region of the person, by means of a personal pump that can be worn on a belt by the worker (72). The sampling flow and the choice of membrane are specified in the analytical method. Particle size-selective devices placed before the collection medium allow the specific fraction of the ambient aerosol to be selected, if necessary. For instance, cyclones can separate the respirable fraction of the dust at airflows as low as a rate of 1.7 L/min, a flow commonly chosen for portable samplers. Aerodynamic small

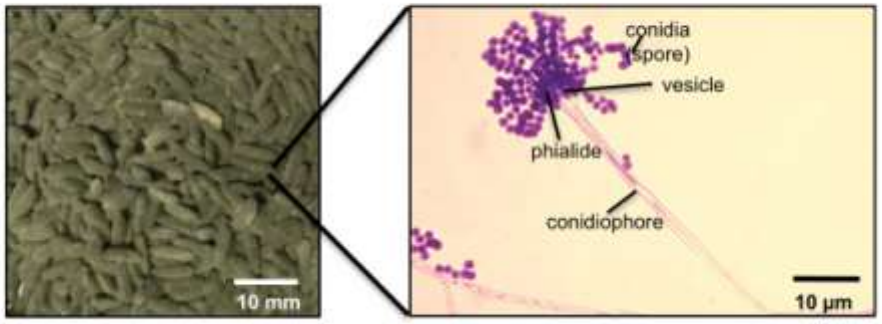
particles are carried by the air flow to the filter contained in a sampling cassette, where they are collected. For the larger non-respirable particles, the settling velocity at flow rate causes them to impact at bottom of the cyclone that is placed prior to a sample filter. A cascade impactor allows the particles to be classified in relation to their size (72). A special sampling method used for allergen sampling is the nasal personal sampler in which dust particles are trapped on an adhesive surface above a tiny filter mounted in the nostrils of a test subject. The strength of the method is that direct sampling from the inhaled air takes place at its high sensitivity. However, there should be no mouth breathing during sampling, while the device may hinder normal nose breathing during physical exercise. There are two types of devices available.

Dust analysis for chemical allergens is done using different techniques including gravimetric analysis, atomic absorption, X-ray diffraction, and microscopy.

Several portable direct-reading instruments are available on the market, mainly for gas and vapor determination. These instruments provide a rapid and continuous reading in real time, and several can collect and process the data. The main utility of these instruments is in the measurement of irritant/oxidant exposures, although paper-tape spectrometric readers and ion mobility meters are used for monitoring some chemical hapten exposures (i.e. diisocyanates). Electrochemical methods and Fourier Transform Infrared (FTIR) spectroscopy are applied mainly for nitrogen oxides, carbon monoxide, oxygen, sulfur dioxide, hydrogen sulfide, etc. and fourier-transform infrared spectrometry for organic and inorganic gases and vapors such as formaldehyde (72). Collection of allergenic chemicals can be non-specific such as filtration, absorption, or employ derivatizing reagent coated collection material. The adsorbent material is then sent to the laboratory for identification and quantitative analysis (73). High-performance liquid chromatography separation, coupled to a mass spectrometer, is commonly used to analyze low-volatile organic compounds like diisocyanates (following derivatization to a stable, non-water reactive form) to achieve sufficient sensitivity to assess ppb levels from the workplace (74). To detect metals inductively coupled plasma-atomic emission spectrometry or mass spectrometry is commonly used. For beryllium or diisocyanates with dermal exposure, human biomonitoring may be needed to provide a better exposure estimate (including both air and dermal exposures)(66, 75). Note, the lymphocyte transformation test is a method useful for detection of cell-mediated sensitization to beryllium which can be detected in a significant proportion of exposed subjects of whom approximately 10% may develop chronic berylliosis (76).

The high molecular weight allergens are generally quantified in the air samples by allergen-specific immunoassays. Procedures for the specific determination of allergens in air samples are available for a wide spectrum of occupational and environmental sensitizing agents, e.g. different grain flour, latex, fish and animal proteins. Advances in sampling systems and laboratory methods for the quantification of these substances have made it possible to determine the environmental concentration of numerous allergens (77-80).

Supplementary Fig. 4: Macroscopic and microscopic view of Aspergillus versicolor a common ubiquitous fungal species, often found in both indoor and outdoor air samples is a common cause of fungal allergy. Both fungal spores and fragments can be components of environmental bioaerosols



Marcoscopic view of *Aspergillus sp.* grow on grains of rice in laboratory conditions

Microscopic view of *A. versicolor* with notable structures labeled.

The figures were kindly provided by Dr. Mark Barnes (CDC/NIOSH)

Supplementary Table 4: Bioaerosol sampling

Bioaerosol sampling

1.1.1.1 In the 5th edition of NMAM is a chapter by Lindsley et al. on "Sampling and characterization of bioaerosols" www.cdc.gov/niosh/nmam (81)

https://www.cdc.gov/niosh/nmam/pdfs/NMAM_5thEd_EBook.pdf.

Examples of bioaerosols include pollen, fungal spores (see Supplementary Figure 4), bacteria and a variety of other aeroallergens. The chapter discusses the various bioaerosol air samplers, and immunoassays and gene-based assays that can be used to characterize and measure exposure. Beezhold et al. (82) examined the prevalence of fungal allergic sensitization in patients with allergic rhinitis or asthma. They noted that 20.5% of this atopic population tested positive for at least 1 fungal extract with Alternaria alternata being the most common. Some of the patients had reactions to antigens from multiple fungal species, and IgE reactivity (Western Blot) was observed for common indoor air species not represented in commercially available test.

Bioaerosol sampling and characterization for allergenic components represent unique and different challenges compared to occupational chemical or protein air assessment. The point

sources for general environmental bioaerosols can be quite diverse and difficult to identify. Both pollens and fungal spores can be transported long distances in the air as evidenced by pollens and fungi collected by bioaerosol samplers attached to aircraft can be sampled at heights up to 2000m above the ground (83). As noted previously, pollen counting is presently a manually intensive endeavor, however automation through use of CCD camera images of the collection filter sample coupled with image analyses was reported to correctly speciate 93% of the pollens identified with a high correlation between manual and automated pollen count, however, the image analysis missed 27.8% of the pollen species (identifying them mainly as unknown) (84).
Potentially future advancements in computer-based image analyses or use of next generation sequencing holds promise to exceed manual methods in bioaerosol identification and quantification.

Sampling

Sampling for allergens is done by passing a known volume of air through a filter using a suction pump. The filters can be of various types (fiberglass, teflon, etc.). It is important to standardize the sampling characteristics such as time and air flow. There are different types of samplers that adapt to different environments. Extraction of allergens: The second stage involves the extraction of soluble allergens from the filter with buffered aqueous solutions. Conditions such as elution buffer used, sample agitation, temperature and elution time should be optimized. For specific aspects of bioaerosol sampling see Supplementary Table 4.

Analytical techniques

Various techniques are used to measure the environmental concentration of aeroallergens. The quantification of some pollen in the air, which have a characteristic morphology, can be performed by optical microscopy techniques using morphological criteria. These techniques, together with those of culture, are also used for the environmental quantification of microorganisms (85). In complex mixtures containing, among other substances, allergenic proteins that cannot be identified morphologically, specific immunoassays must be used (historically RIA and now ELISA techniques) that can be capture (sandwich methods) or competitive (ELISA-inhibition and RAST-inhibition). Identification of the specific allergenic components can be further identified by a combination of Western Blot immunoassay using the allergen-specific IgE from patients' sera along with ultra-high performance liquid chromatographic mass-spectral proteomic analysis. The interpretation of the results is difficult since the allergic pathology is triggered by a sensitization mechanism that depends on the individual susceptibility and there is a high heterogeneity in the immunogenic properties of the allergens. Added to this diversity is the fact that the concentration needed to sensitize an individual is greater than that which causes symptoms to an already sensitized individual (86). Therefore, environmental assessments are useful to demonstrate exposure, assess the effectiveness of interventions aimed at reducing this exposure and establish dose-response relationships, without there being general limit values for all allergic individuals.

Assessment of dermal exposure to LMW agents

Dermal exposure to LMW agents can cause type 1 sensitization associated with respiratory and/or skin allergies. In the occupational setting air monitoring may be insufficient in assessing potential allergen exposures.

There are no standardized, validated assessment methods for dermal exposure to chemicals able to cause asthma. Several published diisocyanate studies have assessed dermal exposure (as reviewed by (67)). Free diisocyanate has been measured by means of skin wipe testing, and derivatizing reagent-impregnated patches, cotton sleeves or gloves. Skin tape stripping, followed by hydrolysis of the epidermal samples to measure total diisocyanate as the diamine hydrolysis product, has also been employed (87). Liljelind et al. (2010) using this method demonstrated methylene diphenyl diisocyanate penetration into the skin and exposure not only to the hands, but also to the forehead (presumably thought touching their face or head with their hands, or possibly though aerosol deposition). Assessment of dermal exposure using derivatizing reagent impregnated patches or gloves provides an estimate of the immediate exposure, while epidermal tape stripping may represent a more semi-cumulative exposure to the skin area sampled since the stratum corneum turnover is 7-18 days dependent on age and body region (88). Dermal exposure to LMW allergens may also occur from commercial and personal care/cosmetic products outside the workplace. Isocyanate based foams and glues are readily available for consumer use, organic acid anhydrides are found in finger nail hardeners, acrylates in finger nail glues, and formaldehyde releasing agents found in cosmetic/consumer products can expose consumers to formaldehyde during their use.

Exposure assessment with biomonitoring (biomarkers of exposure)

Unlike air measurements, the human biomonitoring measures the levels of environmental chemicals or their metabolites in easily accessible body fluids and tissues and reflects all routes of uptake - oral, dermal, inhalative - and all relevant sources (biomarker of exposure). Many allergens (also almost all low-molecular weight allergens) can thus be measured in body fluids. The exposure to isocyanates can be measured with urinary isocyanate biomarkers in urine (in µg/L with urinary creatinine included as confounder) (89). Also hemoglobin or albumin adducts serve as useful biomonitoring methods reflecting long term past exposure to isocyanates (58, 90). For other LMW allergens (which act as haptens or as reactive agents forming new antigenic determinants with host proteins), chemical analyses of the hapten hydrolysis products like i.e. methyl hexahydrophthalic anhydrides from blood or urine has been employed (55, 91).

For some antigens, measurements of specific IgG can provide useful information about past exposure to allergens. In general, measurement of antigen-specific IgG from a patient's serum can enable the estimation of the extent of exposure to both low and high molecular agents. This approach was successfully applied for dicarboxylic anhydrides, diisocyanates and wheat proteins (56, 92-96). Antigen-specific IgE has also been used. Although specific-IgE has greater specificity with respect to asthma/allergy, it is of lower sensitivity as a marker of exposure.

Questionnaires

Questionnaires are important tools in both epidemiological and clinical studies. Associations between specific jobs, job tasks and allergen exposure can be determined and asthma-specific job exposure matrixes developed. Significantly, assessment of past exposure(s), including potential high transient/acute, potentially sensitizing events may be identified. Le Moual et al. (97) surveyed 14,151 adults from the general population and found increased risk of asthma from jobs or specific agents including "industrial cleaning agents, latex, flour, highly reactive chemicals and textiles." Examples of questionnaires that have been used in occupational asthma studies can be found in the section below. In a northern European Cohort among 13,284 subjects Lillienberg and coworkers were able to identify new-onset asthma among workers exposed to plant allergens, epoxy acrylates, peak irritant exposure and cleaning agents by using a asthma specific JEM aligned to northern European working conditions (98).

Safety data sheets (SDS)

SDS (previously called MSDS) of products used in the workplace should always be checked in detail when a patient reports symptoms consistent with an allergic reaction. Indicated hazard statements and precautionary measures according to REACH regulations are important information on potential health risks. However, it should be taken into consideration that the risk depends on concentrations, combined effects of hazardous substances and individual susceptibility factors.

c) Questionnaires, and medical histories by experts

Questionnaires are useful screening tools in epidemiological studies. Several epidemiological questionnaires are in current use. Important questions refer to specific exposure and association of symptoms to work may be used in clinical diagnosis. In the clinical setting questionnaires that identify symptoms of wheeze and/or shortness of breath which improve on days away from work or on holiday have a high sensitivity, but relatively low specificity for occupational asthma.

There is general agreement that medical histories taken by experts have high sensitivity, but their specificity may be lower (9).

Assessment of past exposures should be done by a generalist and a specialist. A generalist should elicit exposures in high-risk occupations, in which asthma should be assumed to be occupational unless excluded by objective tests. The workers reported from population studies to be at increased risk of developing asthma include; bakers, chemical workers, cleaners, cooks, electrical and electronic production workers, farm workers, food processors, forestry workers, healthcare workers, laboratory technicians, mechanics, metal workers, painters, plastics and rubber workers, storage workers, textile workers, waiters, welders and wood workers. A specialist should identify specific exposures, with a systematic occupational history, scrutiny of exposure documentation such as SDS, internal reports and industrial hygiene measurements from the industry. The failure to find a sensitizer on a SDS should not preclude the diagnosis of

occupational asthma, as many sensitizers are not regularly listed, particularly those in low concentration, those that are only present in certain circumstances, such as when heated and those given non-specific titles such as preservative, biocide, fragrance, resin etc.

Questionnaire examples

Danish Asthma questionnaire (in Danish): Danish Asthma questionnaire (in Danish)

Asthma questionnaire (X. Baur): see Appendix 1 in (23).Validation of an asthma questionnaire for use in healthcare workers/A survey of asthma in health professionals by Delclos et al.:

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2078145/bin/oenvmed_63_3_173__index.html

Supplementary material to: Integrated diagnostic approach for occupational and environmental industrial allergens

Special diagnostic aspects regarding work-related asthma

Individual diagnostic steps: overview

The diagnosis of allergic asthma necessitates knowledge on a) the exposure to an environmental allergen, b) demonstration of a causal association between exposure and asthma symptoms (associated with airflow limitation), c) an increase of non-specific bronchial hyperresponsiveness (NSBHR) and/or d) induction of airway inflammation (e.g. sputum eosinophils) plus the identification of a specific IgE-mediated allergic reaction to the allergen. Due to negative allergological findings for some special environmental agents, pathogenetic mechanisms are however still not always elucidated; examples include most cases of isocyanate and potroom asthma.

In the clinical setting repeated exposure-related symptoms such as wheezing, cough, shortness of breath, sneezing, nasal hypersecretion or congestion, urticaria should initiate a conclusive diagnostic work-up to identify the causative agent and objectively confirm its clinical role. Occupational allergens conform to the general definition of allergens.

The initial diagnostic step is a detailed and qualified medical and environmental history. A questionnaire filled in by the patient (see examples above) and supplemented by the trained physician in an interview is recommended.

This is followed by physical examination and allergological tests by use of common as well as in the individual case relevant specific environmental allergens. Skin prick testing and sIgE tests mostly represent alternative approaches. They may supplement each other because relevant occupational and environmental industrial allergens are frequently not available in both methods and because there exist some differences in specificity and sensitivity. Testing should comprise all allergens relevant in the individual case from the general and home environment, as well as

the workplace. Specific IgE measurement would gain a much greater diagnostic impact if the still existing shortcomings mentioned in the above chapters will be overcome.

Spirometry (99) is required in all patients with suspected asthma (Supplementary Fig. 5a). It is used as a main instrument for monitoring lung function longitudinally during surveillance, also during measurement of non-specific bronchial hyperresponsiveness (NSBHR), in specific inhalation challenge and in workplace challenge test. Asthmatic patients may show normal lung function parameters when seen in the outpatient department. There is also a role for measurement of airway resistance or specific conductance to monitor lung function when a patient's FEV_1 cannot be reliably obtained.

Spirometry monitoring (serial spirometry measurements) during work shifts and on days off over a 2 week period (regularly done 4 times daily) has been shown to be superior to measurement of cross-work shift changes alone. Computerized interpretation of measured lung function parameters has been shown to be useful, e.g. by OASYS software (100).

Specific inhalation challenge test (SIC) (101) is commonly regarded as a reference method for diagnosing asthma in doubtful cases (supplementary Figure 5a, c), especially when far-reaching consequences from the test result are expected, for example change of work or environment, immunotherapy (see below). However, SIC is complex and time consuming and necessitates special expertise, equipment and trained personnel.

It should be mentioned that none of the diagnostic tests used alone in diagnosis of WRA yields a sufficiently high combination of sensitivity and specificity for replacing SIC. Spirometry, increased diurnal variation in PEF, sputum eosinophilia and exhaled nitric oxide may all help to confirm WRA, but may all be normal in individuals with WRA confirmed by specific challenge tests. However, also a negative SIC test in a worker with otherwise good evidence of occupational asthma is not sufficient to exclude the diagnosis due to the possibility of false negative SIC, e.g. due to too low concentration used, too short exposure time or a long interval since last exposure (102). So, since none of those tests taken alone allows for diagnosis WRA with a sufficient level of confidence the prudent WRA diagnostic approach should combine well designed SIC with all available functional and immunologic tests.

Case history

All patients with asthma and COPD should undergo a detailed case history and being asked whether their symptoms improve on days without exposure, e.g. in case of suspected WRA when they are away from work or on holidays.

Non-invasive methods to assess airway inflammation

Non-invasive methods used to assess and monitor airway inflammation in asthma comprise induced sputum, exhaled nitric oxide (FeNO; see Supplementary material for details), exhaled breath condensate (EBC). The first methods are very useful for monitoring the inflammatory

activity in the airways. However, their definite place in practical diagnosis still has to be established.

Exhaled nitric oxide (FeNO) is a non-invasive tool for assessing airway inflammation in asthma (103). NO is produced by various cells in the lung either resident or recruited during the inflammatory process. Exhaled NO is generally measured on line, the subject blowing directly into the analyzer that provides immediate breath NO level data. Portable analyzers are also available. It is also possible to have a remote breath collection into inert bags, with subsequent analysis. NO concentration is increased in the exhaled air from patients with asthma and decreased by corticosteroid therapy. There is a positive correlation between FeNO and sputum eosinophils in asthma. In clinical practice, evaluation of FeNO was equivalent to sputum eosinophils in diagnosing asthma (104).

Some studies have investigated the role of FeNO in assessing OA, but with inconsistent results (105-107). It has been suggested that measurement of FeNO can be used to indicate the development of airway inflammation accompanying late asthmatic re- actions after SIC in patients with normal or slightly increased basal NO levels (107). However, the usefulness of FeNO in the investigation of OA may be limited by factors affecting its determination, such as therapy with inhaled steroids and smoking (103). Although the measurement of FeNO is totally non-invasive, quick and relatively simple to perform, more data are needed to determine whether FeNO is useful in diagnosing work-related asthma. In the clinical setting, a finding of normal exhaled nitric oxide fraction cannot be used to exclude asthma.

Increase in sputum eosinophilia (between 6 and 24 hours) and in FeNO (between 6 and 48 hours) are typical in early as well as late asthmatic reactors after exposure to high- or low-molecular weight occupational agents. Their addition to the other diagnostic methods (see above) and to lung function monitoring increases the sensitivity and specificity of the diagnostic procedure and of surveillance of endangered subjects (108-111). Exposure to occupational agents in asthmatics not sensitized to the agents do not induce airway inflammation and do not lead to significant changes of the parameters of these methods. Not all subjects suffering from WRA exhibit sputum eosinophilia, however most of these negative ones have less NSBHR. It should be mentioned that sputum eosinophilia is also present in occupational non-asthmatic eosinophilic bronchitis, and to a lower degree frequently also in allergic rhinitis subjects.

Measurement of non-specific airway responsiveness (NSBHR) (112)

This method by means of challenge with methacholine is part of the initial diagnostic work-up in case of suspected asthma. It can also be measured before and after a period of work exposure and during SIC. Normal NSBHR does not exclude OA.

Serial lung function testing. In case of allergic asthma, exposure to the causative agent should result in a measurable decline in lung function during and mostly after exposure. This is best performed by a portable spirometer (113). Measuring FEV1 during periods of work and away from work may document a causal relationship between occupational exposure and airflow reduction]. 4 readings/day are required, although 8 readings a day allows shorter records, with usual exposure on work days and no changes in treatment on days away from work. A 3-week record containing at least 3 periods off work are suitable for most forms of analysis]. Note: New

handheld electronic spirometers storing the whole flow volume curves and automatically checking for reproducibility and other quality criteria (e.g. ATS/ERS 2005; (99)) will allow easy quality control and provide additional parameters.

Specific inhalation test (SIC)

This test should be performed in a specially designed laboratory. Controlled concentrations of the suspected causative agent are administered in controlled concentration and dose either by the inhalation chamber method or by a nebulizer. A recently published statement comprises the state of the art knowledge and describes the methods in detail (114).

The purpose of SIC is to explore through a direct observational approach the causal relationship between exposure to occupational agents and asthma. Agents causing immunological OA should be able to induce after a clinically silent induction phase the development of the characteristic features of asthma, including "variable airflow limitation, nonspecific bronchial hyperresponsiveness (NSBHR) and airway inflammation.

The major rationale for performing challenges with occupational agents is that this experimental approach remains the most reliable procedure to document "organ-specific responsiveness to an occupational agent" in a given individual (115) In individuals with suspected sensitizer-induced OA, conducting SICs (where available) is suggested when the diagnosis or causative agent remains equivocal with clinical history, assessment of non-specific bronchial hyperresponsiveness, and immunological tests (116). In the rapidly industrialized world, this clinical need has been increasingly common with numerous new emerging chemical compounds. SIC is the best method of confirming a "new" and/or "particularly chemical" specific cause of occupational asthma when workplace measurements are not possible or specific IgE measurements are not available, as in the case for many LMW agents (117). SIC tests could also be included as the final confirmatory step in high-risk workplaces, and to assess the efficacy of preventive measures and protective devices.

For further details see the ERS handbook of procedures for specific inhalation challenge testing in the diagnosis of occupational asthma (118).

The sensitivity and specificity of SIC are high but not easy to quantify; the method is currently used as the reference standard for the diagnosis of occupational asthma (101). A systematic review on OA due to HMW and LMWt agents (LMW), exhibited a sensitivity of NSBHR assessment of 79% and 64%, respectively, and a specificity of 51% and 64%, respectively, when its outcome was compared with SIC test results (119). A number of studies using different methods of NSBH test methods reported that NSBH may be present in 5-40% of patients with a positive specific challenge test (120-122)]. PEF monitoring in the work place has a sensitivity of 81-86% and a specificity of 74-86% (120-123). A sputum eosinophils increase of >1% post SIC or workplace exposure may support a diagnosis of occupational asthma when the FEV1 has fallen >20% (124). In a group of 40 workers, exhaled breath nitric oxide (FeNO) levels were significantly increased after a positive SIC test response in those workers with normal or slightly increased pre-challenge FeNO levels (107). SIC testing with diisocyanates was shown to induce an increase in FeNO levels which was more likely in diisocyanate SIC responders compared with non-responders (106).

Workplace challenge test

This test is an attempt to reproduce actual work processes. The aim of a workplace challenge is to make supervised lung function similar to a bronchial provocation testing in the workplace after suitable control measurements have been made without exposure in the workplace on separate days. Measurements of NSBHR, cells in induced sputum, fractional exhaled nitric oxide (FeNO) etc. can be made in conjunction with lung function testing during the workplace challenge. The patient should be exposed to the usual concentrations of the potential causative agent during the challenge that may last up to 4 hours of usual exposure. It is important to ensure that the usual work practices are taking place during the workplace challenge which may not always be easy to achieve.

Although false negative and false positive results cannot totally excluded carefully controlled workplace challenges and SIC come closest to a gold standard test for environmental allergens causing asthma and rhinitis (125-127) A negative test in a subject with otherwise good evidence of respiratory allergy is not sufficient to preclude the diagnosis.

Further details of diagnostic testing in case of suspected asthma are described by Aasen et. al (9).

Allergic rhinitis diagnostic approach

Occupational allergic rhinitis diagnostics includes nasal examinations (anterior rhinoscopy, nasal endoscopy in order to exclude differential diagnoses such as nasal septum deviation, chronic rhinosinusitis, nasal polyposis (128)), assessment of nasal patency and inflammation in nasal secretions. Allergological tests are the same as for asthma (see above). The diagnosis of occupational rhinitis or work-exacerbated rhinitis is based on the causal relationship of rhinitis with specific exposures in the workplace. The gold standard for this is the nasal challenge test by use of the suspected causative agent (Supplementary Figure 6) (2). An alternative is serial measurement of nasal patency (e.g. by peak nasal inspiratory flow, active anterior rhinomanometry, acoustic rhinometry, longitudinal rhinometry for 24 hours, or phase-rhinomanometry) during work shifts and at days off work over a two weeks period, corresponding to the above-mentioned serial measurement of lung function (Supplementary Figure 5d).

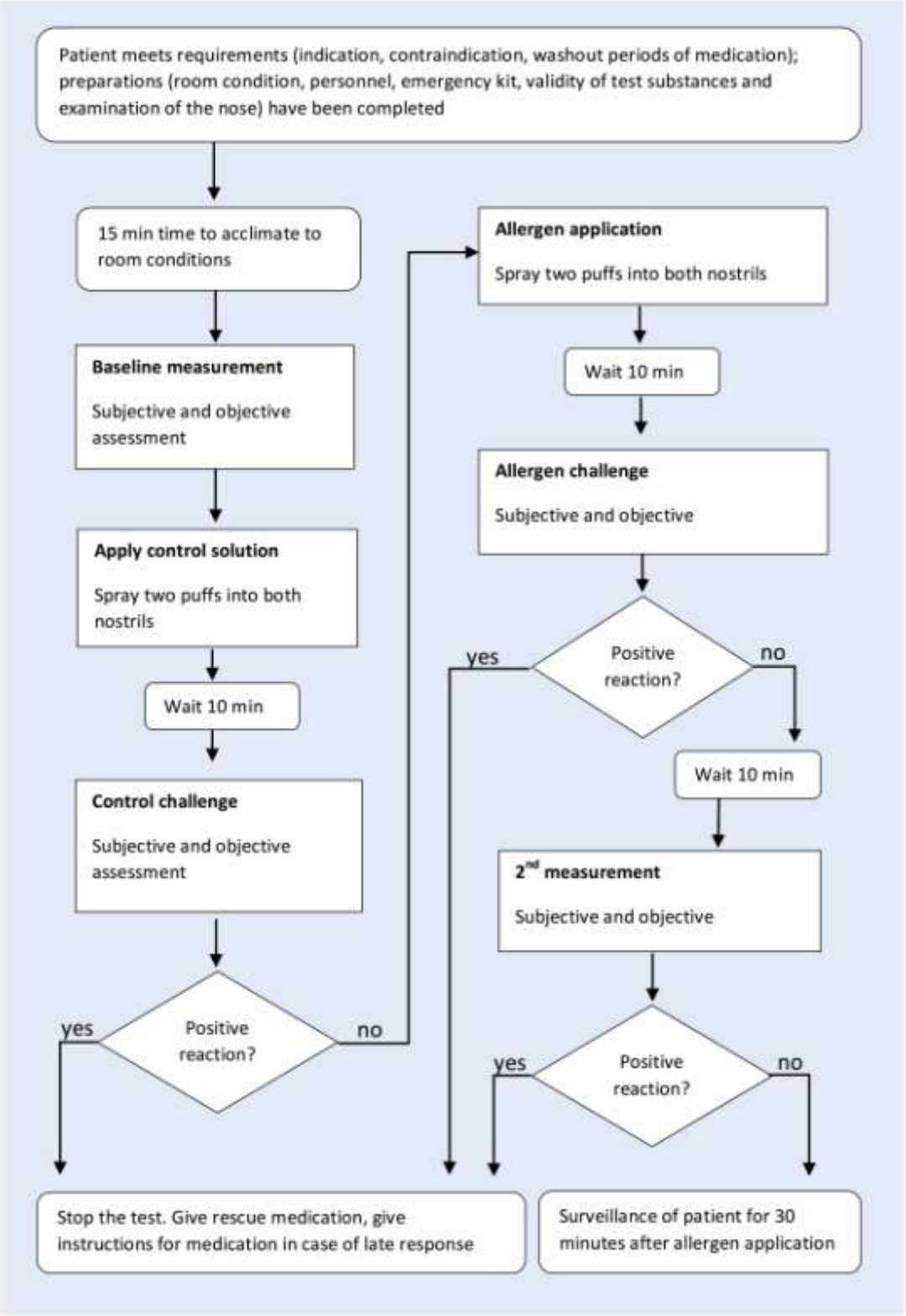
Serial measurement of nasal patency is also recommended if the specific nasal challenge test in the laboratory shows a negative result whereas there is a suggestive history for a cause in the workplace pointing to a specific nasal hyperresponsiveness The nasal hyperresponsiveness can be confirmed by nasal challenges with histamine, methacholine or cold dry air (CDA) (129). There are reports on occupational local allergic rhinitis characterized by work-related rhinitis combined with nasal inflammatory responses but negative allergological tests (130).

Protein contact dermatitis

In case of suspected protein contact dermatitis skin prick testing with the agent obviously being causative is the method of choice. Frequently, there are no commercially available extracts; So, in-house extracts have to be prepared in a standardized manner (see chapter above). An alternative may be skin prick testing or scratch testing by use of the native material, for example raw meat.

Supplementary Fig. 5 In vivo diagnostics: spirometry (a), skin prick testing (b); provocation tests: chamber for SIC testing (c), flow rhinomanometry during nasal provocation test (d)

Supplementary Fig. 6: Algorithm for specific nasal challenge testing (from (2))



Concluding remarks: Points to be emphasized and prospective

IgE-mediated sensitization to airborne occupational allergens is frequently associated with development of allergic rhinitis, conjunctivitis and bronchial asthma. More rarely, dermal contact causes protein contact dermatits (contact urticaria), e.g. among bakers.

If not diagnosed in the early stages, these allergic disorders may become chronic, may lead to unemployment and even disability.

An earlier ERS Task Force involving several of the present task force members focused on the management and preventive aspects of OA, which has become one of the most common occupational lung diseases (9, 117, 131). More than 600 causative agents were identified, around two thirds of which were airway sensitizers (29, 132). Respective diagnostic tests including biomonitoring and ambient monitoring methods for (protein) HMW allergens and/or LMW agents causing asthma such as isocyanates were developed and used (25, 27, 40, 45, 47, 48, 62, 118, 133-142). Every year, new allergenic agents in the worksites are identified (29, 143). However, so far, there exists no critical overview of the performance of the various methods and agents used.

Physiological tests such as lung function monitoring in relation to work exposures or specific challenge testing are used to confirm the work-related nature of the disorder. Specific challenge testing is the reference standard for making the specific diagnosis in individual cases, especially when a new causative agent is suspected (102). A recent consensus statement of the ERS working group summarizes the present state-of-the-art methods for specific inhalation challenges. However, these challenges are cumbersome, expensive, time-consuming and not available at many diagnostic centers; therefore, less invasive alternative methods are required.

At present, occupational asthma sufferers are frequently denied proper compensation because conclusive identification of the cause is regarded as a prerequisite for any settlement. In many cases the diagnostic procedure does not provide sufficient information on the causative relationship. The same is true for preventive measures, which also require identification of causative agents, their sources, and quantitative data.

Nasal challenges confirm in vivo the diagnosis of allergic rhinitis, occupational rhinitis, local allergic rhinitis, and/or nasal hyperreactivity. Recently, a new guideline for nasal allergic challenges (2) and an EAACI position paper on non-allergic rhinitis (144) were published. However, nasal challenges by agents causing occupational rhinitis or nasal hyperreactivity were rarely published. Highly specialized experienced centers of ENT clinics, pulmonology units or occupational medicine institutes focus on diagnoses and differential diagnoses of these different phenotypes.

A recent position paper published by the EAACI group Occupational Allergy and Aerobiology & Air Pollution presents an overview of environmental allergen monitoring (79). The paper focuses on available methods, but does not make specific recommendations for their standardization and quality control; nor does it consider most occupational allergens or new allergenic sources such as genetically-engineered enzymes or other new chemicals with sensitizing properties.

There are a few studies dealing with quantification of airborne allergens at worksites and in the environment. An example is the case of soy hull aeroallergens released during the unloading of soybeans in Barcelona and other ports, which triggered asthma epidemics and caused a number of deaths (24, 28, 145). Other exceptional occupational sensitizers for which measurement data exist include a few wheat flour components (146), *Aspergillus oryzae* a-amylase (147), rat and mouse urinary proteins (148, 149), latex allergens (150), isocyanates (136, 151), and organic acid anhydrides (152). Such data is relevant in the context with reliable specific IgE test results and clinical findings for establishment of management strategies and preventive measures. The successful management of the affected workers involves termination of exposure to the causative agent, whereas reduction does not seem to be effective. Avoidance and especially removal from exposure have been shown to improve the prognosis of workers with occupational asthma (153). To achieve this, the specific causative agents need to be confirmed. Therefore, the same standardized, comparable, reproducible diagnostic tests are needed both for effective state-of-the-art prevention and monitoring of asthma and for obtaining fair compensation for workers suffering from allergy to occupational agents.

The best routine non-invasive methods for determining the precise cause aim to identify specific IgE antibodies in serum of affected subjects. In general, the finding of specific IgE (sIgE) to a well-characterized airborne allergen, in the presence of convincing work-related asthma and/or rhinitis symptoms combined with obstructive functional impairment or contact urticaria upon defined exposure is sufficient to confirm the disorder. However, the absence of allergen-specific IgE is not sufficient to exclude asthma.

It is generally agreed that the measurement of sIgE is not sensitive enough (or even not adequate) for certain sensitizing agents such as LMW allergens (e.g. isocyanates) or the numerous genetically modified proteins present in many workplaces (e.g., enzymes in the detergent and fragrance industry, bakeries and so on).

The in vitro hapten conjugated allergens used in these assays may be responsible for the low sensitivity. What is more, the individual genetic susceptibility and the amount of the total IgE (atopic status) may impact the sIgE result (154).

A wide range of sIgE test methods are currently available on the market and are in use at diagnostic centres in Europe. The heterogeneity of the methods used means that the results are difficult to compare, and most lack standardization and sufficient quality control. Furthermore, there is no standard for the frequently needed in-house allergy tests, since no tests are available at present for many occupational allergens. These shortcomings frequently lead to under diagnosis of respiratory allergies (102). Thus, the immunological diagnosis of allergic

occupational asthma is quite heterogeneous and the results from different centers are hard to compare.

Skin prick testing has been found to be less suitable for these investigations due to the lack of consistency of extracts of occupational agents and also due to legal restrictions (155). Conducting skin-prick testing with “real-world“ products (e.g. food items, occupational agents as used by patients) is considered unethical and illegal by some jurisdictions, on the grounds that this amounts to administering “non-approved“ foreign compounds to human subjects, as if these were drugs. However, when done by people with expertise and with appropriate safety measures, diagnostic skin prick testing with non-approved test agents should be authorized, because the potential health risk of this procedure is minimal in view of the minute quantities of foreign material administered and the very limited time of exposure (as opposed to dermal patch testing, which can induce sensitization); the risk of allowing a potentially sensitized patient to be (or remain) exposed to the offending agent in his/her workplace is much higher, and hence should be ethically and legally much more questioned, than doing skin prick testing.

It is important to design non-invasive evidence-based immunological methods that allow for objective confirmation of sensitization in the diagnosis, prevalence and quantification of the allergenic load as part of the preventive measures in allergic respiratory disorders. Accurate, interdisciplinary interpretation of the data is also crucial to obtaining compensation for diseased workers. Only a positive, sensitive, specific immunological test result indicates sensitization and needs to be followed by enhanced surveillance or further tests to confirm the presence of occupational asthma or another respiratory disorder.

To be useful, sensitivity and specificity tests are needed for each method and allergen, together with proof of any cross-reactivity. Quantifying the inhalable allergenic load in the occupational environment is crucial for early diagnosis, surveillance and introduction of appropriate control strategies (9, 102, 131, 156). Taking into account existing information on air concentrations of occupational allergens when interpreting sIgE findings may improve the performance of the diagnostic test and the identification of the causative allergen. Plausibility checks of the IgE results, as well as controls for false positives and false negatives, are also required. The existing methodological shortcomings need to be resolved in order to improve the diagnosis and prevention of allergic occupational respiratory disorders and thus to ensure that diseased workers obtain fair compensation.

Currently available immunological diagnostic techniques for allergic occupational respiratory disorders lack standardization and an evidence-based approach. In individual cases and worksites considerable uncertainty exists, and there is limited scope for comparing test results between centres. Standardized operating procedures (SOPs) have been found to be helpful defining best laboratory practices and diagnostic cut-offs for the different commercial methods. The clinical diagnostic needs will be addressed by the many experts in occupational asthma, using the practical laboratory-oriented state-of-the art approaches of bio monitoring and ambient monitoring to identify the causative allergens (respective diagnostic schemas will be provided).

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