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# Is the analysis of histamine and/or interleukin-4 release after isocyanate challenge useful in the identification of patients with IgE-mediated isocyanate asthma?

Silke Blindow<sup>a</sup>, Alexandra M. Preisser<sup>b</sup>, Xaver Baur<sup>c</sup>, Lygia T. Budnik<sup>a,\*</sup>

<sup>a</sup> Division of Occupational Toxicology and Immunology, Institute for Occupational and Maritime Medicine (ZfAM), University Medical Center Hamburg-Eppendorf, Germany

<sup>b</sup> Division of Clinical Occupational Medicine, Institute for Occupational and Maritime Medicine (ZfAM), University Medical Center Hamburg-Eppendorf, Germany

<sup>c</sup> Division of Occupational Lung Diseases and Allergy, Institute for Occupational Medicine, Charité-University Medicine Center, Campus Benjamin Franklin, Berlin, Germany

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## ABSTRACT

Isocyanates are a well-known and frequent cause of occupational asthma. The implementation of specific inhalation challenges (SICs) is the gold standard in asthma diagnosis supporting occupational case history, lung function testing, specific skin prick tests and the detection of specific IgE. However, the diagnosis is not always definitive. An interesting new approach, analyses of individual genetic susceptibilities, requires discrimination between a positive SIC reaction arising from IgE-mediated immune responses and one from other pathophysiological mechanisms. Hence, additional refinement tools would be helpful in defining sub-classes of occupational asthma and diagnosis. We used total IgE levels, specific IgE and SIC results for sub-classification of 27 symptomatic isocyanate workers studied. Some mutations in glutathione S-transferases (GSTs) are suspected either to enhance or to decrease the individual risk in the development of isocyanate asthma. Our patient groups were assessed for the point mutations GSTP1\*1105V and GSTP1\*A114V as well as deletions (null mutations) of GSTM1 and GSTT1. There seems to be a higher risk in developing IgE-mediated reactions when GSTM1 is deleted, while GSTT1 deletions were found more frequently in the SIC positive group. Blood samples taken before SIC, 30–60 min and 24 h after SIC, were analyzed for histamine and IL-4, classical markers for the IgE-mediated antigen-specific activation of basophils or mast cells. We suggest that the utility of histamine measurements might provide an additional useful marker reflecting isocyanate-induced cellular reactions (although the sampling times require optimization). The promising measurement of IL-4 is not feasible at present due to the lack of a reliable, validated assay.

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## 1. Introduction

Work-related asthma is one of the most common occupational diseases, comprising occupational asthma and work-

aggravated asthma (Latza and Baur, 2005; Baur et al., 2012). Occupational asthma includes IgE-mediated occupational asthma, occupational asthma arising from specific occupational agents with unknown pathomechanism as well as irritant occupational asthma (Baur et al., 2012), probably with different genetic backgrounds. Patients often are sub-classified as “asthmatic” and “not asthmatic” individuals, “exposed” and “not exposed” workers, or “SIC positive” and “negative” (Mapp et al., 2002; Littorin et al., 2008; Piacentini et al., 2012; Yucosoy et al., 2012). Consequently there is a need for additional criteria to classify the asthma type more exactly.

\* Corresponding author at: Division of Occupational Toxicology and Immunology, Institute for Occupational and Maritime Medicine (ZfAM), University Medical Center Hamburg-Eppendorf, Marckmannstrasse 129 b, Bld. 3, 20539 Hamburg, Germany. Tel.: +49 40 42845 7540.

E-mail address: [L.Budnik@uke.de](mailto:L.Budnik@uke.de) (L.T. Budnik).

Some of the most prevalent causative agents of occupational asthma are isocyanates (Vandenplas, 2011; Baur, 1996; Baur, 2013a; Baur and Bakehe, 2014), chemicals used in many manufacturing areas, including the car, aerospace, metal-working, wood-working as well as mining industries (Allport et al., 2003; Baur, 2003; Pronk et al., 2007; Campo et al., 2010; Vandenplas et al., 2011; Reeb-Whitaker et al., 2013) and in medical supplies or applications (Lambda et al., 1998; Santerre et al., 2005; Cherng et al., 2013). Isocyanate uptake in the workplace is known to occur by inhalation of airborne isocyanate vapors or aerosols generated during the production processes (Vandenplas et al., 1993; Monso et al., 2000; Wisnewski et al., 2000; Budnik et al., 2011; Bernstein et al., 2013), but transdermal uptake is also possible (Schroder et al., 1999; Liu et al., 2000; Wisnewski et al., 2000; Verschoor and Verschoor, 2014). Isocyanates react with several human proteins in vivo, notably albumin, globulins and keratins (Sepai et al., 1995; Wisnewski et al., 2000; Johannesson et al., 2004; Flack et al., 2011; Mhihe et al., 2013), and also form adducts with glutathione (Day et al., 1997; Lange et al., 1999; Wisnewski et al., 2013a; Wisnewski et al., 2013b). The isocyanate-protein adducts are suspected to generate immunogenic epitopes eliciting the production of specific IgE (sIgE) antibodies. However, isocyanate-specific IgE is rarely found in the serum of isocyanate asthma patients (Ott et al., 2007; Hur et al., 2008; Campo et al., 2010; Sharifi et al., 2013), which has even led to the contemplation that isocyanate asthma is a model of IgE-independent asthma (Mapp et al., 2005; Jones et al., 2006). Nevertheless, skin prick tests and/or the measurement of specific IgE antibodies using isocyanate-albumin conjugates were shown to be useful in differentiating between IgE-mediated and non-IgE-mediated reactions to isocyanates (Beach et al., 2007; Wisnewski and Jones, 2010; Budnik et al., 2013). In vivo, IgE has a multitude of capturing partners, like cell-bound or soluble high and low affinity receptors (Conrad, 1990; Kinet, 1999; Kraft and Kinet, 2007; Gould and Sutton, 2008; Acharya et al., 2010; Dehlink et al., 2011; Platzer et al., 2011). Hence, free specific IgE antibodies might be present, but below analytical detection levels (Dehlink et al., 2010). Moreover, the results of direct specific IgE measurements or indirect detection methods like skin prick or basophil activation tests (BAT) are strongly dependent on the quality of the artificially produced isocyanate-albumin conjugate used as screening antigen (Ye et al., 2006; Campo et al., 2007; Wisnewski et al., 2010). The direct measurement of sIgE-triggered mediator releases from FcεRI bearing cells into the blood after SIC could avoid the pitfalls of using artificial antigens, if the released concentrations would be sufficiently above the background. FcεRI-bearing cells are basophils, mast cells, eosinophils and some antigen presenting cells (Von Bubnoff et al., 2003; Prussin and Metcalfe, 2006). Basophils are most suitable to identify the presence of specific IgE indirectly via the release of e.g. histamine, IL-4, IL-13 and LTC<sub>4</sub> after antigen binding (Lichtenstein and Bochner, 1991; Gibbs et al., 1996; Kita et al., 1999). We considered histamine and IL-4 as potential markers, and analyzed their release after the isocyanate challenge in this study. In addition to the presence of specific IgE antibodies the total IgE (tIgE) level might provide a good premise for the development of the type-I allergy. Usually, a value above 100 U/mL is used as an indicator of type-I allergies (Wittig et al., 1980; Zetterstrom and Johansson, 1981),

although 20 U/mL was already proposed as a negative threshold value in 1981 (Zetterstrom and Johansson, 1981).

We analyzed 27 isocyanate-exposed patients that underwent a total of 41 inhalation challenge tests as a part of an expert opinion examination. According to the SIC and the tIgE results, the patients were subdivided into four groups and compared with regard to IL-4 and histamine releases after SIC. We hypothesized that these subdivision criteria would result in more homogeneous results of genetic studies. A number of enzymatic antioxidants, including glutathione S-transferases (GSTs), manganese superoxide, dismutase and microsomal epoxide hydrolase may play a protective role in redox imbalance in the lung, contributing to isocyanate asthma, DA (Yucesoy et al., 2012). Mutations of glutathione S-transferase (GST) genes have been reported to be associated with asthma generally (Aynacioglu et al., 2004; Kleeberger and Peden, 2005; Minelli et al., 2010), but have also aroused interest during the investigation of isocyanate asthma (Piirila et al., 2001; Mapp et al., 2002; Wikman et al., 2002; Liu and Wisnewski, 2003; Broberg et al., 2008; Littorin et al., 2008; Broberg et al., 2010; Bernstein, 2011; Yucesoy et al., 2012), leading to differing conclusions about their impact on the individual predisposition. We performed a pilot analysis comprising mutations in GSTM1, GSTT1 and GSTP1 genes, and compared the mutation frequencies between the four groups of isocyanate exposed workers.

## 2. Materials and methods

### 2.1. Subjects

A total of 27 isocyanate-exposed patients were examined with regard to detailed clinical and occupational case history, lung function and sensitization to common environmental allergens using skin prick tests and the measurement of total IgE antibodies. Morphometric data (height, weight, smoking status, type and length of exposure and interim since last exposure) was obtained by questionnaire. The 41 challenges were conducted (2007–2013) with the following diisocyanates: hexamethylene diisocyanate (HDI, 12), methylene diphenyl diisocyanate (MDI, 11), toluene diisocyanate (TDI, 6), naphthalene diisocyanate (NDI, 5), isophorone diisocyanate (IPDI, 5) and HDI-containing hardening agents (2). In addition, individual sensitizations to isocyanates were examined by means of isocyanate-specific skin prick tests (SPTs) and the measurements of specific IgE (sIgE) antibodies.

The study was approved by the local ethics committee and the institutional review board. The clinical examination was performed in the course of the social security case examination. All patients gave signed written agreement to use their samples and data.

### 2.2. Skin prick tests (SPTs)

All patients underwent SPTs with a series of 20 common allergens arising from: *Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*, dog and cat hair and dander, *Plantanus spec.*, weed mixture, *Artemisia vulgaris* and *Ambrosia artemisiifolia* (ragweed), *Parietaria spec.*, *Alternaria alternata*, *Cladosporium herbarium*, and *Aspergillus fumigatus* (ALK-Abelló Arzneimittel GmbH, Hamburg, Germany), 3 mixtures of pollen

from early flowering trees, *Lepidoglyphus destructor* (HAL Allergy GmbH, Düsseldorf, Germany), *Acarus siro* and *Tyrophagus putrescentiae* (Allergopharma Jochen Ganzer KG, Reinbek, Germany), Plantago spec. (Bencard Allergie GmbH, Munich, Germany) and natural latex from *Hevea brasiliensis* (0.01 mg/mL, in-house preparation). Negative and positive controls (solvent alone and 0.01 mg/mL histamine solution, ALK-Abelló Arzneimittel GmbH) were included. The allergens were gently pricked onto the skin surface of the volar side of the forearm. The results were read 20 min later and regarded as positive if at least one wheal with a diameter > 3 mm developed and the negative control resulted in no or a smaller wheal. Specific isocyanate SPTs were performed in an analogous manner using isocyanate–human serum albumin (HSA) conjugates (MDI–HSA TDI–HSA, NDI–HSA or HDI–HSA), prepared as freeze-dried powder by Bayer AG, Leverkusen, Germany as a special request from XB, according to the published method (Baur, 1983; Baur et al., 1984). The conjugates were dissolved in 0.9% sterile NaCl solution with 0.4% phenol as stabilizing agent (also contained in the commercially available allergen preparations listed above) until a final concentration of 1 mg/mL of the respective isocyanate conjugate was achieved.

### 2.3. NSBHR (non-specific bronchial hyper-responsiveness)

The protocol for NSBHR testing has been described before (Baur et al., 1998). Briefly, the inhalation challenge involved serial measurements of FEV<sub>1</sub> with progressively increasing doses of methacholine, starting with 0.015 mg in 500 mL air, followed by doubling the dose, which was achieved by doubling the operational time of the nebulizer, i.e., the aerosol quantity up to a cumulative methacholine dose of 0.471 mg. The methacholine was aerosolized by a timer-controlled fine droplet jet nebulizer (Pari provocation test II, Pari GmbH Starnberg, Germany). The reaction was defined as positive if FEV<sub>1</sub> decreased at least 20% after a cumulative application of ≤ 0.3 mg methacholine (Baur et al., 1998).

### 2.4. Specific inhalation challenge (SIC)

The SIC method has been described in detail elsewhere (Budnik et al., 2011). The patients were exposed to isocyanates in an exposure chamber with a maximum concentration of 5.5 ppb up to 120 min, starting with 2.5 ppb for the first 10 min. FEV<sub>1</sub> was measured before and then 10 min, 30 min, 60 min, 2 h, 3 h, 5 h and 7 h from the start of the exposure. The SIC result was considered positive when FEV<sub>1</sub> decreased at least about 20%. We analyzed blood serum samples taken before SIC, 30–60 min after the challenge and 24 h after SIC for histamine, IL-4, total IgE and total protein content. Specific IgE was measured using the serum sample taken before SIC. If initially, sIgE value was found to be positive, all samples of the patient were analyzed to follow up any changes due to the challenge.

### 2.5. DNA extraction

EDTA-blood was taken for DNA extraction, which was performed using the QIAamp Blood Mini Kit (Quiagen) according to the manufacturer's protocol. The DNA content and purity were measured using a Nanodrop 2000c (ThermoFisher Scientific). Two extracts from each donor were prepared

independently on different days and were analyzed separately in all genotyping experiments.

### 2.6. GSTP1 genotyping: point mutation affecting the amino acid 105 (GSTP1\*1105V)

The GSTP1 exon 5 105 ile → val polymorphism was determined by PCR followed by a restriction analysis on the basis of the method of Harries et al. (1979). DNA samples were amplified with the primers 5'-ACCCCAGGGCTCTATGGGAA-3' (forward) and 5'-TGAGGGCACAAGAAGCCCT-3' (reverse) (Sigma). Since the extracts contained both mitochondrial and nuclear DNA the concentration of the latter was unknown. Therefore different volumes of the templates were used to obtain similar amounts of PCR products as assessed by agarose gel electrophoresis (not shown). The PCR amplification was performed using 50 µL of the Taq PCR Mastermix (Quiagen) in a final volume of 100 µL, providing a final concentration of 1.5 mM MgCl and 200 µM of each dNTP. The optimal primer concentration was established experimentally (not shown) and found to be 0.75 µM (both forward and reverse). Amplification was performed with an initial denaturation step at 95 °C for 5 min, followed by 30 cycles with denaturing at 94 °C for 30 s, annealing at 64 °C for 30 s, elongation at 72 °C for 60 s and a final extension step at 72 °C for 10 min. The annealing temperature was specified by Harries et al. (1979) as 55 °C, but was found to be optimal at 64 °C in our experiments (not shown). The annealing temperature should be 5–10 °C beyond the melting temperatures of the primers, which were 68.8 and 70.3 °C according to the manufacturer (Sigma). The PCR products were stored frozen at –20 °C until further processing. 20 µL of the PCR products containing roughly the same amounts of amplified DNA, as assessed in agarose gel electrophoresis, was digested with 20 U of BsmAI (New England Biolabs, Beverly, MA) using the provided “10× NE buffer 4” at a final concentration of 1× (containing 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9 at 25 °C). Controls were prepared by replacing the enzyme with DEPC-treated water. All samples were incubated at 55 °C for 2.5 h, which was found experimentally (not shown) to be sufficient for a complete digestion. Controls without the addition of enzyme were run simultaneously with the restriction experiments. After the addition of 12.5 µL gel loading buffer (Invitrogen), the fragment lengths were analyzed on an agarose gel (150 × 140 mm<sup>2</sup>, 3.5%, 80 V/0.1 A) containing ethidium bromide (0.5 µg/mL). 1× TBE buffer was used to prepare the gels and as running buffer. When the BsmAI restriction site was present, the PCR product consisting of 176 bp was digested into two lengths of 91 and 85 bp. Agarose gel electrophoresis of the digested PCR products showed individuals homozygous for the wild type (ile/ile) as one band of 176 bp. Extracts containing a heterozygous distribution of this single nucleotide polymorphism (ile/val) resulted in three bands of 176, 91 and 85. Homozygotes (val/val) showed two bands of 91 and 85 bp (Fig. 1).

### 2.7. GSTP1 genotyping: point mutation affecting the amino acid 114 (GSTP1\*A114V)

To find potential GSTP1 exon 6 polymorphisms, resulting in an exchange of amino acid 114 (ala → val), also a PCR reaction

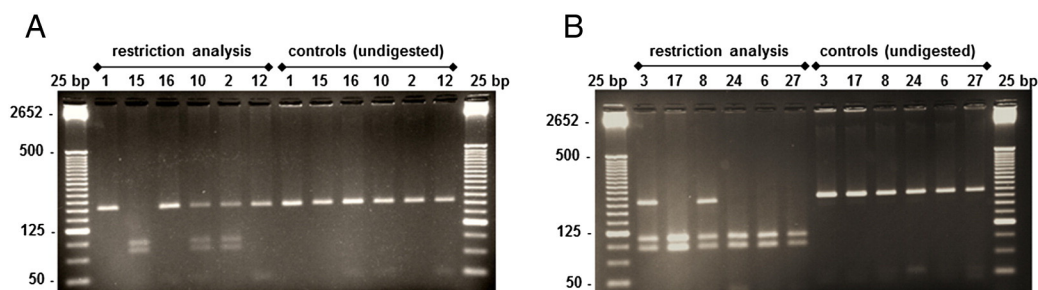


**Fig. 1.** Analysis of single point mutations (SNPs) of the GSTP1 gene. Undigested controls show roughly comparable DNA amounts. Fragments of the DNA standard differ in 25 bp between 50 and 500 bp. A: GSTP1\*1105V: The digested samples of patients 1, 16 and 12 show the homozygous wild type (ile/ile), by giving one fragment of 176 bp. The homozygous mutation (val/val, patient 15) results in two fragments (91 and 85 bp). Samples of the heterozygous type (ile/val, patient 10 and 2) show all three fragments after digestion. B: GSTP1\*A114V: the wild type gene features a restriction site for Cac8I, resulting in two fragments (113 and 93 bp) after digestion. After point mutation the restriction site is lost, resulting in one large fragment (206 bp) after digestion. Patients 17, 24, 6 and 27 are homozygous wild type, while patients 3 and 8 are heterozygous wild type. Homozygous mutants were not found.

followed by a restriction analysis was performed as described by Board et al. (1998). The sequences of the primers (Sigma) were 5'-GTTGTGGGGAGCAAGCAGAGG-3' (forward) and 5'-CACAAATGAAGTCTTCGCTCC-3' (reverse).

Preliminary experiments (not shown) revealed optimal PCR conditions with primer concentrations of 0.3  $\mu$ M (both forward and reverse primers) and an annealing temperature of 62 °C. (The melting temperatures of the primers were indicated as 70.3 and 68.8 °C by the manufacturer). The other terms of the PCR procedure and the storage of the PCR products (217 bp) were the same as described above except for the different annealing temperature. The digestion experiments were carried out as described above except for using 20 U of the enzyme Cac8I (New England Biolabs) with a digestion temperature of 37 °C for 2.5 h (checked out in preliminary experiments, not shown). The results of the restriction analysis were visualized by performing agarose gel electrophoresis as described above. The wild type DNA contains two Cac8I restriction sites, resulting in two larger fragments of 113 and 93 bp lengths and a small fragment (11 bp). After point mutation (wild type: GCGGGC, mutated sequence: GTGGGC), a large fragment (206 bp) as well as a small fragment (11 bp) is found. Heterozygous samples show both of these fragments (Fig. 1).

## 2.8. GSTM1 and GSTT1 genotyping

The absence (null mutants) or presence of GSTM1 and GSTT1 encoding genes was examined using a multiplex PCR method. CYP1A1 (Cytochrome P450-1A1) was used as reference gene to prove the presence of sufficient amounts of genomic DNA. 3 primer pairs were added simultaneously to 25  $\mu$ L Taq PCR Mastermix (Quiagen) and varying amounts of template DNA in the following pairwise final concentrations (found out in preliminary experiments, not shown) in a total volume of 50  $\mu$ L: 0.8  $\mu$ M of GSTM1 primers, 0.6  $\mu$ M of GSTT1 primers and 0.3  $\mu$ M of CYP1A1 primers. The primer sequences were: 5'-GAACTCCTGAAAGCTAAAGC-3' and 5'-CTTGGGCTCAAATATACGGTGG-3' (GSTM1), 5'-TTCCTTACTGCTCCTCACATCTC-3' and 5'-TCACCGATCATGGCCAGCA-3' (GSTT1), 5'-GAAC TGCCACTTCAGCTGTCT-3' and 5'-CAGCTGCATTGGAAGTGCTC-3' (CYP1A1). The sequence of the GSTM1-forward primer differs in one base from the corresponding one in Krause and Müller (2002) due to a check of the DNA sequence of the GSTM1 gene in the NCBI

database. Preliminary tests resulted in an optimal annealing temperature of 57 °C in our experimental design ((Krause and Müller, 2002): 64 °C). The PCR procedure (cycle scheme) as well as the agarose gel electrophoresis was performed as specified above. The control band (CYP1A1) must appear at 312 bp. If a GSTM1 gene is present in the DNA of a donor there will be a band at 218 bp, while the presence of a GSTT1 gene results in a band at 459 bp (Fig. 2). This multiplex PCR method does not discriminate between heterozygous and homozygous wild type.

## 2.9. Total protein content

The total protein content was measured using the Pierce® BCA Protein Assay Kit (No. 23225, Thermo Scientific, Schwerte, Germany) according to the manufacturer's instructions for the microplate procedure. Each serum sample was diluted 1:50 twice, then each dilution was measured in duplicate ( $n = 4$ ). The absorbance was measured at 560 nm. The standard curve was fitted with a quadratic model as specified.

## 2.10. Total IgE ELISA

We measured the total IgE serum concentrations using the RIDASCREEN® Total IgE ELISA (No. A0141, R-Biopharm AG,

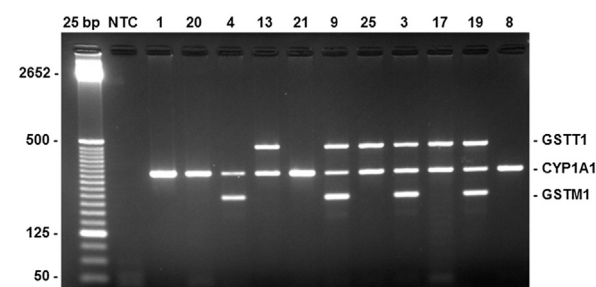


**Fig. 2.** Analysis of GSTM1 and GSTT1 gene deletions. The control gene product of CYP1A1 (cytochrome P450 family; 312 bp) must be present in all samples to ensure sufficient DNA template. If the corresponding gene is present in a DNA sample, a band with a size of 469 bp representing GSTT1 and/or 218 bp representing GSTM1 appeared. The absence of a band implies a homozygous null type. Heterozygosity is not reflected in this multiplex analysis. Fragments of the DNA standard differ in 25 bp between 50 and 500 bp.

Darmstadt, Germany), which generates similar results as the ImmunoCap method (according to a comparison of the total IgE values of all samples at time point 0, thus before SIC; data not shown). All serum samples were analyzed in duplicate using according to the manufacturer's protocol. Point-to-point regression was performed to fit the calibration curve (5–1000 IU/mL). Two provided controls with known results were used to normalize the measured results from different plates.

To compare total IgE (tIgE) values of all serum samples (before and after challenge), the results were expressed as IU IgE per mean serum protein content (see Section 3.2), in order to exclude differences due to water uptake or loss. According to the manufacturer's instructions, the presence of an IgE-mediated allergy is unlikely when the total IgE concentration is below 20 IU/mL, is possible for ranges between 20 and 100 IU/mL and is probable if tIgE is above 100 IU/mL. In accordance with these specifications, supported by some publications and our own findings (also discussed below), our patients were grouped into “isocyanate allergy (type I) unlikely” if tIgE was found to be <20 IU/mL and “isocyanate allergy (type I) possible” if serum concentrations above 20 IU/mL were measured.

#### 2.11. Isocyanate-specific antibodies (sIgE, sIgG)

Isocyanate-specific antibodies were analyzed using a fluorescence enzyme immune assay (semi-automatic ImmunoCap100, Thermo Fisher Scientific Inc., Waltham, MA 02454, USA), using commercially available ImmunoCAPs for TDI (k75), MDI (k76) and HDI (k77) from the same manufacturer. CAPs for the detection of NDI-specific IgE were prepared as follows: NDI-HSA (Bayer AG) was dissolved in phosphate buffered saline pH 7.4 with a final concentration of 1–5 mg/mL. Biotin-X-X-NHS (Sigma-Aldrich Chemie GmbH, Munich, Germany) was prepared in DMSO with concentration of 20 mg/mL, and added to the allergen solution in a ratio of 5:1. After 2 h of incubation on a magnetic stirrer the biotinylated NDI-HSA was purified using a PD-10 column (Sigma-Aldrich Chemie GmbH). The grade of biotinylation was estimated via an HABA/Avidin assay (Sigma-Aldrich Chemie GmbH) and should be 1. Then 25 µg of the biotinylated NDI-HSA in a volume of 50–100 µL was bound to one prewashed Streptavidin ImmunoCAP with an incubation time of 30 min. After another washing step the NDI-CAPs were ready to use.

For the detection of sIgE or sIgG, a seven-point dose-response calibration was performed monthly, while in each daily measurement two curve controls and one additional supplied control was included, as indicated in the manufacturer's instructions. The in-house prepared NDI-CAPs were also evaluated using patient sera with known values in repeated measurements during method validation. Serum-specific IgE is expressed in kilo unit per liter (kU/L) calibrated using the WHO reference of human serum IgE (1 kU = 2.4 ng/mL). For ImmunoCAP-specific IgE, the limit of detection (LOD) is 0.02 kU/L for IgE and 0.2 mg/L for IgG. Values above 0.35 kU/L for sIgE and 3 mg/L for sIgG are regarded as positive. CAP classes (Phadia/Thermo Fisher) of sIgE are: 0 = < 0.35 U/mL, 1 = 0.35–0.70 U/mL, 2 = 0.70–3.50 U/mL, 3 = 3.50–17.5 U/mL, 4 = 17.5–50 U/mL, 5 = 50–100 U/mL, and 6 = >100 U/mL.

#### 2.12. Histamine ELISA

The histamine assay (No. HIS00, WESAMIN GmbH & Co. KG, Winsen, Germany) was performed according to the manufacturer's instructions. Samples were acylated in duplicate and two aliquots of each preparation ( $n = 4$ ) were transferred onto the ELISA plates. The standard curve (0.15–25 ng/mL) was achieved by plotting the optical density (OD) against the logarithms of the indicated concentrations and applying a four-parameter logistic-curve fitting method. The concentrations of the four values of each sample and subsequently the mean concentration as well as the standard deviation were calculated. The sensitivity of the assay was denoted by the manufacturer to be 0.02 ng/mL.

The variation coefficients (CVs) of the concentrations were calculated by dividing the standard deviation of a quadruplicate through its mean and multiplying the result with 100. All CVs were averaged.

The results were expressed as ng histamine per mean serum protein content (see Section 3.2).

#### 2.13. IL-4 ELISA

Serum samples were analyzed in triplicate using a commercially available IL-4 ELISA (No. EH3IL4, Thermo Scientific, Schwerte, Germany). The sensitivity was declared to be <2 pg/mL. As discussed later in the Results section, we performed higher dilutions (1.56–50 pg/mL instead of 10.24–400 pg/mL) of the calibrator solution in order to achieve an increased sensitivity and reproducibility at low concentrations. Standard concentrations were plotted against the corresponding OD values and fitted using a linear regression model. Another IL-4-ELISA (No. KHC0044, Life Technologies GmbH, Darmstadt, Germany), which was indicated to detect IL-4 doses < 0.27 pg/mL was also applied. This assay has been calibrated against the WHO reference preparation 88/656 (NIBSC, Hertfordshire, UK, EN6 3QG), which is a recombinant protein. The assay was performed according to the manufacturer's instruction, and a four-parameter logistic algorithm was used to fit the calibration curve as indicated. All samples were analyzed in triplicate. Mean concentrations as well as standard deviations were calculated. The variation coefficients (CVs) of the triplicate values were calculated and averaged as described above.

#### 2.14. Statistics

GraphPad Prism (Version 6.0) was used to perform multiple t-tests in order to analyze the statistical significance of the individual histamine release tests and to calculate odds ratios for group comparisons. If any of the four values of a contingency table is zero, which sometimes was the case, GraphPad Prism adds 0.5 to all values before calculating odds ratios to avoid dividing through zero.

### 3. Results

The clinical data is shown in Table 1. Due to the low number of patients we did not evaluate the results of our genetic or protein analysis in consideration of sex, age, smoking status, obstructive ventilation pattern at baseline and NSBHR.

**Table 1**

Clinical features of 27 isocyanate-challenged subjects, grouped by SIC, sIgE, and total IgE results. Group 1: SIC plus sIgE positive, tIgE > 20 IU/mL; group 2: SIC positive, tIgE < 20 IU/mL; group 3: SIC negative, tIgE > 20 IU/mL; group 4: SIC negative, tIgE < 20 IU/mL.

PY: pack years; mo: month; NSBHR: non-specific bronchial hyperresponsiveness (methacholine test); n: number; n.d.: not done; TDI: toluene diisocyanate; MDI: diphenylmethane diisocyanate; NDI: naphthalene diisocyanate HDI: hexamethylene diisocyanate; IPDI: isophorone diisocyanate.

<sup>1</sup>3 years jobless in between; <sup>2</sup>increase of temperature (1 °C) during SIC; <sup>3</sup>sSPT positive; \*hardener; early = early asthmatic response; dual = dual asthmatic response.

| Group | Patient No. | Sex/age | Workplace                           | Cigarette smoking (PY) | Isocyanate exposure (mo) | Work-related symptoms (mo) | Interim (mo) | Obstruction (baseline testing) | NSBHR | SIC (n) | Isocyanate used | SIC result           |
|-------|-------------|---------|-------------------------------------|------------------------|--------------------------|----------------------------|--------------|--------------------------------|-------|---------|-----------------|----------------------|
| 1     | 1           | m/31    | Piano-cabinet prod.                 | 5                      | 66                       | 48                         | 5            | —                              | +     | 1       | MDI             | Early (MDI)          |
|       | 2           | m/48    | QC-Laboratory                       | 0                      | 70                       | 48                         | 0            | —                              | —     | 2       | IPDI, TDI       | Neg. <sup>3</sup>    |
|       | 3           | m/51    | PU production                       | 0                      | 10                       | 10                         | 8            | —                              | +     | 1       | NDI             | Dual (NDI)           |
|       | 4           | m/65    | PU production                       | 0                      | 180                      | 156                        | 32           | —                              | +     | 2       | TDI, MDI        | Dual (TDI)           |
|       | 5           | m/33    | Paint & coating production          | 15                     | 148                      | 36                         | 40           | —                              | —     | 2       | IPDI, HDI       | Early (IPDI)         |
|       | 6           | m/55    | Grinding workshop                   | 0                      | 73                       | 72                         | 12           | +                              | n.d.  | 1       | NDI             | Dual (NDI)           |
|       | 7           | m/57    | Repair of machines                  | 4                      | 396                      | 408                        | 0            | —                              | +     | 1       | MDI             | Dual (MDI)           |
|       | 8           | f/53    | Spray painting                      | 0                      | 144                      | 120                        | 0            | —                              | —     | 2       | HDI, TDI        | Rhinitis (HDI + TDI) |
| 2     | 9           | m/40    | Spray painting                      | 2                      | 156                      | 2                          | 9            | —                              | —     | 3       | HDI, TDI, HDI*  | Early (TDI)          |
|       | 10          | m/27    | Filler & painting work <sup>1</sup> | 5                      | 72                       | 108                        | 10           | —                              | +     | 2       | HDI, IPDI       | Rhinitis (HDI)       |
|       | 11          | m/47    | Glue machine                        | 0                      | 132                      | 126                        | 0            | —                              | +     | 1       | MDI             | Early (MDI)          |
|       | 12          | m/49    | PU production                       | 30                     | 132                      | 30                         | 8            | +                              | n.d.  | 2       | MDI, NDI        | Dual (NDI)           |
| 3     | 13          | m/46    | Carpentry + painting                | 15                     | 529                      | 156                        | 60           | —                              | +     | 1       | HDI             | Neg.                 |
|       | 14          | m/43    | Automobile painting                 | 23                     | 288                      | 96                         | 45           | —                              | —     | 2       | HDI, MDI        | Neg. <sup>2</sup>    |
|       | 15          | m/36    | Spray painting                      | 30                     | 82                       | ?                          | 22           | +                              | n.d.  | 1       | HDI             | Neg.                 |
|       | 16          | m/34    | Painting workshop                   | 0                      | 156                      | 84                         | 0            | —                              | —     | 1       | HDI             | Neg.                 |
| 4     | 17          | m/35    | Metall-coating unit                 | 14                     | 5                        | 5                          | 9            | —                              | +     | 1       | MDI             | Neg.                 |
|       | 18          | m/34    | Aircraft assembly                   | 0                      | 209                      | 36                         | 24           | +                              | n.d.  | 1       | HDI             | Neg.                 |
|       | 19          | m/49    | Vehicle body repair                 | 0                      | 48                       | 48                         | 4            | —                              | —     | 2       | HDI, HDI*       | Neg.                 |
|       | 20          | m/61    | Spray painting                      | 18                     | 132                      | 36                         | 11           | —                              | +     | 1       | TDI             | Neg.                 |
|       | 21          | f/49    | PU final processing                 | 29                     | 529                      | 28                         | 13           | —                              | +     | 2       | MDI, NDI        | Neg.                 |
|       | 22          | m/37    | Production of plastics              | 11                     | 141                      | 72                         | 7            | —                              | —     | 2       | NDI, MDI        | Neg.                 |
|       | 23          | m/50    | Spray painting                      | 0                      | 342                      | 46                         | 46           | —                              | +     | 2       | IPDI, HDI       | Neg.                 |
|       | 24          | m/47    | Carpenter workshop                  | 3                      | 151                      | 72                         | 9            | —                              | —     | 1       | MDI             | Neg.                 |
|       | 25          | f/30    | Chemical laboratory                 | 0                      | 116                      | 44                         | 16           | —                              | —     | 2       | TDI, IPDI       | Neg.                 |
|       | 26          | m/54    | Spray painting                      | 0                      | 420                      | 60                         | 55           | —                              | —     | 1       | HDI             | Neg.                 |
|       | 27          | f/43    | Magnet coating                      | 2                      | 18                       | 18                         | 2            | —                              | —     | 1       | MDI             | Neg.                 |

### 3.1. SIC, specific SPT, specific IgE

27 patients underwent 43 specific inhalation challenges altogether using different isocyanates according to those used in their workplace occupational situation (Table 1). 11 respiratory responses and one borderline response (increasing temperature) were registered. 3 of the 11 SIC-positive patients (all of the three with a total IgE concentration > 20 IU/mL serum) also showed positive SPT to the corresponding isocyanate-HSA-conjugate; one of them was also found to express specific IgE in the range from 0.70 to 3.5 IU/mL (CAP class 2) against the lung-reaction causing isocyanate as well as against another isocyanate. One single patient (no. 2) expressed specific IgE (both CAP class 1: from 0.35 to 0.70 IU/mL) for TDI- and HDI-HSA-conjugates, but showed neither positive SIC results with TDI and IPDI nor any positive specific SPT reaction (Table 2).

### 3.2. Total protein content (BCA) in serum

The mean protein content of all samples was 66 mg/mL (range: 47.8–81.5 mg/mL). The protein concentration of 5 patient serum samples differed between 20 and 30% from the initial sample before SIC (not shown). 18 other samples showed differences of 10–20% from the sample taken before

challenge (not shown). In order to eliminate influences of water uptake or loss between the sampling, the results of the serum histamine, IL-4 and total IgE analyses were normalized to the mean total protein content of 66 mg/mL (“normalized mL”, mL(N)).

### 3.3. Total IgE (tIgE) content and the sensitization against common allergens

The mean CV of the measured optical density (OD) values of all plates ranged between 2.1 and 5.9% while the CV of the calculated concentrations (IU/mL) of the duplicates was between 2.6 and 8.4% (mean of a plate), indicating that the assay worked well. Total IgE values before challenge were related to the sensitization against common allergens as estimated in SPTs (Tables 2 and 5). 7 patients expressed more than 100 IU/mL, 5 (71.4%) of them were sensitized. The serum of another 8 patients contained tIgE in the range of 21–100 IU/mL, thereof 4 (50.0%) with a positive SPT. The remaining 12 patients had less than 20 IU/mL IgE and none of them (0%) was found to be sensitized. Interestingly tIgE values decreased 30–60 min after SIC in all patients serum with initial values above 100 IU/mL(N), independently of the SIC results (Fig. 3).

This decrease after SIC was also observed for the specific IgE values in two patients. The observed decrease was not

**Table 2**

Results of GSTP1, GSTM1 and GSTT1 mutation analysis related to SIC / sIgE results to total IgE as measure of type-I reaction probability. Group 1: SIC/sIgE positive, tlgE > 20 IU/mL; group 2: SIC positive, tlgE < 20 IU/mL; group 3: SIC negative, tlgE > 20 IU/mL; group 4: SIC negative, tlgE < 20 IU/mL; W/W: wild type, W/M: heterozygous mutant, M/M: homozygous mutant, +: gene is present, once at least, 0: gene deletion; PM: point mutation <sup>1</sup>SIC neg, but sensitized (sIgE!), <sup>2</sup>before SIC, <sup>3</sup>wheat only 2 mm in diameter; <sup>4</sup>CAP classes: 0 = <0.35, 1 = 0.35–0.70, 2 = 0.70–3.5, 3 = 3.5–17.5, 4 = 17.5–50.0, 5 = 50.0–100, and 6 = >100 IU/mL; <sup>5</sup>defined as positive, if the sIgG result – referred to the isocyanate used in the SIC – was above 3 mg/mL <sup>6</sup>The number of positive SPTs is indicated.

| Group No. | Patient No. | SPT <sup>6</sup><br>(Common allergens) | total IgE <sup>2</sup><br>(IU/mL)<br>(ELISA) | SIC              | Isoc.-<br>spec.<br>SPT | Isoc.-<br>sIgE<br>(CAP) <sup>4</sup> | Isoc.-<br>sIgG<br>(CAP) <sup>5</sup> | Glutathion S-transferases |             |    |    |
|-----------|-------------|----------------------------------------|----------------------------------------------|------------------|------------------------|--------------------------------------|--------------------------------------|---------------------------|-------------|----|----|
|           |             |                                        |                                              |                  |                        |                                      |                                      | P1<br>PM105               | P1<br>PM114 | M1 | T1 |
| 1         | 1           | 6+                                     | 472                                          | MDI +            | MDI, HDI               | MDI(2), HDI(1)                       | –                                    | W/W                       | W/W         | 0  | 0  |
|           | 2           | 4+                                     | 375                                          | (–) <sup>1</sup> | –                      | TDI(1), HDI(1)                       | +                                    | W/M                       | W/W         | 0  | 0  |
|           | 3           | 3+                                     | 365                                          | NDI +            | –                      | –                                    | –                                    | W/M                       | W/M         | +  | +  |
|           | 4           | –                                      | 163                                          | TDI +            | –                      | –                                    | –                                    | W/M                       | W/W         | +  | 0  |
|           | 5           | –                                      | 101                                          | IPDI +           | –                      | –                                    | –                                    | W/M                       | W/M         | 0  | +  |
|           | 6           | 2+                                     | 86                                           | NDI +            | –                      | –                                    | –                                    | W/W                       | W/W         | +  | +  |
|           | 7           | 4+                                     | 72                                           | MDI +            | MDI, HDI               | –                                    | +                                    | W/W                       | W/W         | 0  | +  |
|           | 8           | 2+                                     | 67                                           | HDI, TDI +       | (HDI) <sup>3</sup>     | –                                    | +                                    | W/M                       | W/M         | 0  | 0  |
|           | 9           | 2+                                     | 32                                           | TDI +            | –                      | –                                    | +                                    | W/M                       | W/W         | +  | +  |
| 2         | 10          | –                                      | 17                                           | HDI +            | –                      | –                                    | +                                    | W/M                       | W/W         | +  | 0  |
|           | 11          | –                                      | 13                                           | MDI +            | –                      | –                                    | –                                    | W/M                       | W/W         | +  | +  |
|           | 12          | –                                      | 12                                           | NDI +            | –                      | –                                    | +                                    | W/W                       | W/W         | +  | 0  |
| 3         | 13          | 1+                                     | 784                                          | –                | –                      | –                                    | –                                    | W/M                       | W/W         | 0  | +  |
|           | 14          | 1+                                     | 117                                          | –                | –                      | –                                    | +                                    | W/W                       | W/W         | 0  | +  |
|           | 15          | 4+                                     | 93                                           | –                | –                      | –                                    | +                                    | M/M                       | W/M         | 0  | +  |
|           | 16          | 3+                                     | 54                                           | –                | –                      | –                                    | +                                    | W/W                       | W/W         | 0  | +  |
|           | 17          | 6+                                     | 50                                           | –                | –                      | –                                    | +                                    | W/M                       | W/W         | 0  | +  |
|           | 18          | –                                      | 36                                           | –                | –                      | –                                    | –                                    | W/M                       | W/W         | 0  | +  |
| 4         | 19          | –                                      | 16                                           | –                | –                      | –                                    | +                                    | M/M                       | W/W         | +  | +  |
|           | 20          | –                                      | 16                                           | –                | –                      | –                                    | +                                    | W/W                       | W/W         | 0  | 0  |
|           | 21          | –                                      | 14                                           | –                | –                      | –                                    | +                                    | W/W                       | W/W         | 0  | 0  |
|           | 22          | –                                      | 12                                           | –                | –                      | –                                    | +                                    | W/W                       | W/W         | +  | +  |
|           | 23          | –                                      | 7                                            | –                | –                      | –                                    | –                                    | M/M                       | W/W         | 0  | +  |
|           | 24          | –                                      | 5                                            | –                | –                      | –                                    | +                                    | W/M                       | W/W         | +  | +  |
|           | 25          | –                                      | 5                                            | –                | –                      | –                                    | +                                    | W/W                       | W/W         | 0  | +  |
|           | 26          | –                                      | 5                                            | –                | –                      | –                                    | –                                    | W/W                       | W/W         | +  | +  |
|           | 27          | –                                      | 3                                            | –                | –                      | –                                    | +                                    | W/W                       | W/W         | +  | +  |

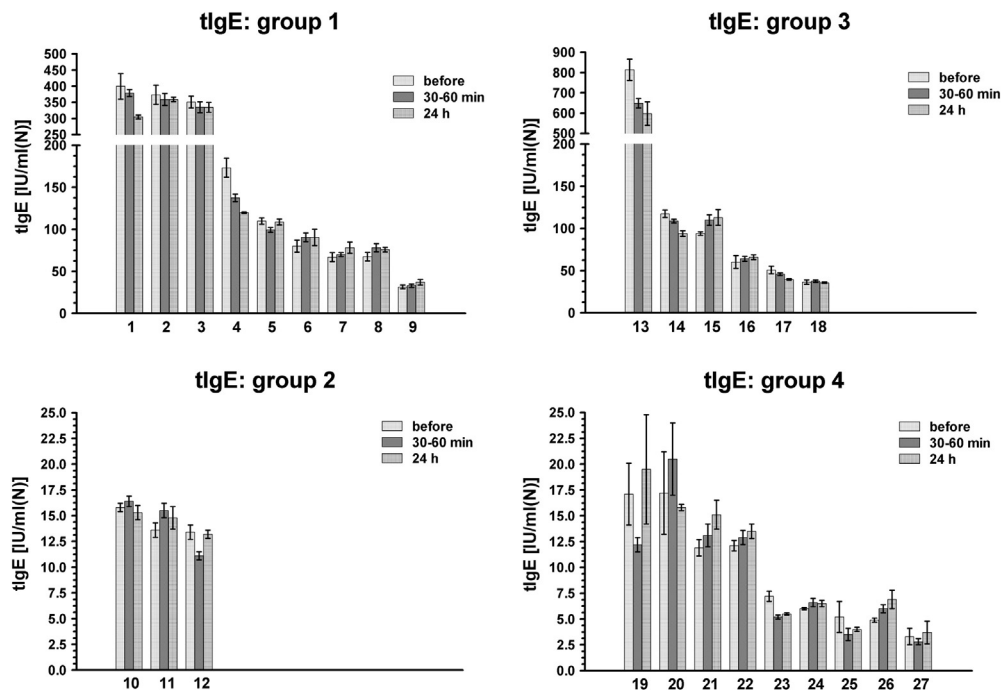


**Fig. 3.** Changes in the total serum IgE content 30–60 min and 24 h after SIC. All total IgE concentrations were normalized to the mean serum protein content of 66 ng/mL.

dependent on the isocyanate used in the SIC and the specificity of the IgE (Fig. 4).

### 3.4. Classification of the challenged patients according to SIC, slgE and total IgE

According to the relationship between total IgE levels below 20 IU/mL and the absence of sensitization against common allergens as estimated in SPT it was assumed that patients with total IgE values below 20 IU/mL are unlikely to develop IgE-mediated reactions (against isocyanate-albumin adducts, formed during the uptake of isocyanates). We considered that there might be a different genetic susceptibility within the patient group showing positive SIC reactions after isocyanate challenge in relation to the total IgE expression. Thus, we divided the examined patients according to the presence of specific reactions toward isocyanates (positive SIC, presence of specific IgE antibodies, positive SPT) and the total IgE concentration either side of the threshold value of 20 IU/mL. Accordingly 4 groups were created (Tables 1 and 2): 1. specific reactions shown, total IgE levels > 20 IU/mL, 2. specific reactions shown, total IgE levels < 20 IU/mL, 3. no specific reactions, total IgE levels > 20 IU/mL, and 4. no specific reactions, total IgE levels < 20 IU/mL. In addition, both the genetic as well as the protein analyses were evaluated for these four groups (data is shown in Figs. 3 and 5 as well as in Tables 2 and 3).

### 3.5. Slightly enhanced histamine release in SIC positive patients with tIgE > 20 IU/mL

The mean CV of all OD values was 4.7% and of the histamine concentrations was 13.8%. The results of the included low and high controls were also found to be within the indicated ranges, so that the performance of the histamine assay was reliable.

The histamine concentrations of the serum samples were expressed as ng histamine per normalized mL, according to the total protein content. After having sub-grouped the patients according to SIC, slgE results and total IgE values as described above, we found slightly enhanced histamine values 30 min after SIC in 7 of 9 patients (78%) in group 1. In groups 2 and 3,

no histamine increase was found at that time point. In group 4, one out of nine subjects (11%) showed increased histamine release (Fig. 5). Unexpectedly, just the patient (no. 1) expressing the most isocyanate-specific IgE antibodies showed no histamine release at that time point.

### 3.6. Undependability of the available assays impedes the IL-4 analyses

Since IL-4 ELISAs have very different ranges of calibrators and diverging statements concerning normal values, we tested two assays; one with calibrators between 10.24 and 400 pg/mL and a denoted sensitivity of <2 pg/mL (Thermo Scientific, Schwerte, Germany). We have to report high variation coefficients both for OD values (around 28%) and calculated concentrations (~37%). 15 of our values seemed to be below the lowest standard (10.24 pg/mL), but above 2 pg/mL, thereof 5 samples with values between 10.1 and 16.5 pg/mL. We tried several changes in the assay procedure with regard to incubation times and temperatures as well as the dilution of the standards to enhance the sensitivity and the performance of the assay, but without satisfactory results. Also dilutions of sample with a measured value above 10 pg/mL produced inconsistent results. According to the assay manufacturer, healthy individuals have no detectable IL-4 in plasma or serum.

We also tested another IL-4 assay (Pierce Biotechnology, Rockford, IL 61105 USA) with a sensitivity of <0.27 pg/mL and calibrators between 0.39–12.5 pg/mL. Although the ODs of the calibration curve were in the requisite range and the controls also gave results as indicated, no IL-4 was detected in any of our serum samples.

### 3.7. Genotyping

The analysis of two separate DNA extracts of each patient performed in all genotyping assays resulted in no discrepancies. The incidence of a single point mutation (SNP) at the triplet coding for amino acid 105 of GSTP1 in the whole group was: 12/27 (44.4%) individuals were homozygous wild type (Ile/Ile) and 44.4% were heterozygous (Ile/Val), while 3/27 (11.1%) were found to be homozygous mutant (Val/Val).

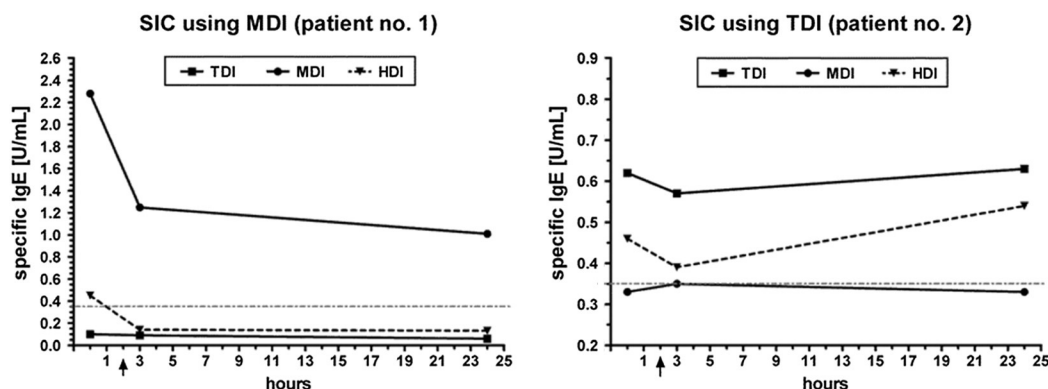


**Fig. 4.** Trends of the specific IgE serum content after SIC. 2 of 27 patients were found to express detectable isocyanate-specific IgE (slgE). slgE values below 0.35 U/mL are considered as negative (CAP class 0). In both patients, the slgE values corresponding to the isocyanate used for the challenge decreased 30–60 min after the end of SIC (→). But during the same time, other slgE types as well as the total IgE content (see Fig. 3) decreased.

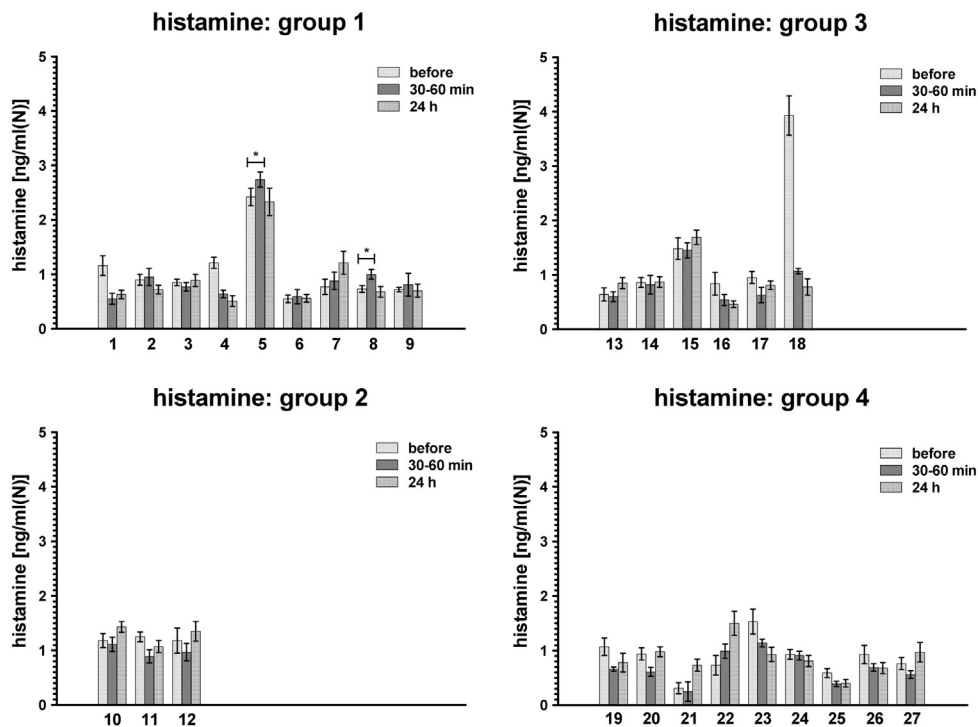


**Fig. 5.** Histamine release after specific inhalation challenge (SIC). 6 of 9 patients from group 1 show slight histamine release 30–60 min after SIC, which was only statistically significant ( $p < 0.05$ ) in 2 cases. Patient no. 8 was classified as SIC-positive due to rhinitis caused by both isocyanates used in SIC. The second challenge (not shown) caused a more significant histamine release after 30–60 min than the first, which is shown in this figure. In the other groups, there was no histamine release 30–60 min after SIC except for patient no. 22 of group 4, who was unlikely to react isocyanate-specifically.

The SNP analysis for GSTP1\*A114V resulted in 23/27 (85.2%) homozygous wild type and 4/27 (14.8%) heterozygous individuals. No homozygous mutation was found. 12/27 (44.4%) of the patients showed neither a SNP affecting amino acid 105 nor 114 of GSTP1. Interestingly, all four individuals (100%) who showed a heterozygous mutation with regard to the GSTP1 114 ala → val polymorphism also had at least one mutated allele resulting in an exchange of amino acids at position 105.

Homozygous deletions (null mutants) of the GSTM1 gene were found 15 times (55.6%), and 8 times (29.6%) for the GSTT1 gene. In 5/27 individuals (18.5%), both GSTM1 and GSTT1 genes were missing. 9/27 (33.3%) subjects possessed at least one allele (+) of both GSTM1 and GSTT1. Null mutations of GSTM1 combined with the wild type of GSTT1 appeared 10 times (37.0%), while the GSTT1 null mutation combined with the presence of GSTM1 was found only 3 times (11.1%).

**Table 3**

Distribution of GST mutations in the four patient groups. Total numbers and percentages (%) related to the patient number of the respective group are stated. Group 1: SIC/sIgE positive, tlgE > 20 IU/mL; group 2: SIC positive, tlgE < 20 IU/mL; group 3: SIC negative, tlgE > 20 IU/mL; group 4: SIC negative, tlgE < 20 IU/mL. W/W: wild type, W/M: heterozygous mutant, M/M: homozygous mutant, +: gene is present, once at least, 0: gene deletion. Publications of Ginsberg et al. (2009) and Littorin et al. (2008) summarized data from several studies about GST mutation distributions in Caucasians, shown in this table (partly adapted; a: GSTP1\*I105V, B: GSTM1 + GSTT1, c: GSTM1, d: GSTT1, and e: GSTP1\*I105V + GSTP1\*A114V).

|                  |            | Results of the study |          |          |          |           | General distribution                |                                                           |
|------------------|------------|----------------------|----------|----------|----------|-----------|-------------------------------------|-----------------------------------------------------------|
|                  |            | Group 1              | Group 2  | Group 3  | Group 4  | Total     | Ginsberg et al.                     | Littorin et al.                                           |
| Patient          | Number (n) | 9 (100)              | 3 (100)  | 6 (100)  | 9 (100)  | 27 (100)  | 739 <sup>a</sup> /1230 <sup>b</sup> | 10,514 <sup>c</sup> /5577 <sup>d</sup> /1591 <sup>e</sup> |
| Common allergens | (SPT pos.) | 7 (77.8)             | 0 (0)    | 5 (83.3) | 0 (0)    | 12 (44.4) | –                                   | –                                                         |
| GSTP1*I105V      | W/W        | 3 (33.3)             | 1 (33.3) | 2 (33.3) | 6 (66.7) | 12 (44.4) | (42.0–51.0)                         | (43.0)                                                    |
|                  | W/M        | 6 (66.7)             | 2 (66.7) | 3 (50.0) | 1 (11.1) | 12 (44.4) | (39.4–51.0)                         | (45.0)                                                    |
|                  | M/M        | 0 (0)                | 0 (0)    | 1 (16.7) | 2 (22.2) | 3 (11.1)  | (6.5–9.1)                           | (12.0)                                                    |
| GSTP1*A114V      | W/W        | 6 (66.7)             | 3 (100)  | 5 (83.3) | 9 (100)  | 23 (85.2) | –                                   | (84.0)                                                    |
|                  | W/M        | 3 (33.3)             | 0 (0)    | 1 (16.7) | 0 (0)    | 4 (14.8)  | –                                   | (16.0)                                                    |
|                  | M/M        | 0 (0)                | 0 (0)    | 0 (0)    | 0 (0)    | 0 (0.0)   | –                                   | –                                                         |
| GSTM1            | +          | 4 (44.4)             | 3 (100)  | 0 (0)    | 5 (55.6) | 12 (44.4) | (47.0)                              | (44.0)                                                    |
|                  | 0          | 5 (55.6)             | 0 (0)    | 6 (100)  | 4 (44.4) | 15 (56.6) | (53.0)                              | (56.0)                                                    |
| GSTT1            | +          | 5 (55.6)             | 1 (33.3) | 6 (100)  | 7 (77.8) | 19 (70.4) | (80.0)                              | (87.0)                                                    |
|                  | 0          | 4 (44.4)             | 2 (66.6) | 0 (0)    | 2 (22.2) | 8 (29.6)  | (20.0)                              | (13.0)                                                    |

The results of the mutation analyses, the information concerning positive SPT to common allergens, total IgE values (before challenge), SIC and SPT results as well as the detection of specific antibodies with regard to the patient subgroups are summarized in Table 2. In Table 3 the distribution of GST mutations of the four patient groups is expressed as total numbers and percentages (%).

It is apparent that all six patients of group 3 (type-I allergies against common allergens, but non-sensitized to isocyanates) had GSTM1 null mutations and at least one GSTT1 wild type gene. In contrast, all 3 subjects of group 2 (sensitized to isocyanates, but unlikely IgE-mediated) have at least one GSTM1 wild type allele. None of all 12 patients with a total IgE value below 20 IU/mL (groups 2 and 4) shows a point mutation in GSTP1\*A114V, while in group 1 there is an incidence of 33.3% (3/9 patients) and in group 3 of 16.7% (1/6 patients). With respect of GSTP1\*I105V, the mutation rates of groups 1, 2 and 3 are identical, while group 4 exhibits double the percentage of wild type (Table 3). Conversely, the highest amount of homozygous GSTP1\*I105V mutations (2 of 9 patients) is found in group 4. Although we observed differences in the mutations for the four groups, these are not statistically significant differences as calculated from odds ratios (Table 4), since the patient numbers of the groups are too low.

Table 5 shows odds ratios calculated from the distribution of genotyping results and results of SPT (common allergens) and total IgE, respectively. The results are also expressed as percentages of mutations related to SPT or tIgE. We found a statistically significant relationship concerning the GSTM1 null mutation, which is coupled to a higher risk of developing IgE-mediated immune responses expressed as total IgE value above 20 IU/mL. 73% patients with more than 20 IU/mL tIgE in their blood serum are GSTM1 null mutant, in contrast with only 33% patients with GSTM1 deletions and less than 20 IU/mL tIgE. Expressed in percentages (75/40), a GSTM1 deletion also leads to a higher prevalence of positive SPT against common allergens. According to the calculated percentages, mutations in GSTP1\*A114V also seem to increase the risk to develop IgE-mediated reactions, either seen in SPT or in the tIgE expression. A deletion of GSTT1 was found significantly more often in the SIC positive patients (group 1 + 2) compared with the SIC negative patients (group 3 + 4).

## 4. Discussion

### 4.1. IgE antibodies

It is generally agreed that the presence of specific IgE (sIgE) is indispensable for the development of a type-I allergy. The prevalence of isocyanate-specific IgE in a fraction of asthmatic isocyanate-exposed workers was shown by numerous studies, which were summarized in some reviews (Baur et al., 1994; Bernstein and Jolly, 1999; Wisniewski, 2007). The measurement of free sIgE was generally found to be specific but not sensitive enough (Bernstein et al., 2002; Campo et al., 2007; Ott et al., 2007; Palikhe et al., 2011). The artificial antigens used in the assays can be one cause of the low sensitivity; another one might be the low amount of free IgE, as discussed below. The half-life of IgE is the topic of many discussions. It must be differentiated between the half-life of individual IgE molecules, either free or bound, and the maintenance of a certain IgE level.

By injecting  $^{14}\text{C}$ -labeled IgE into patients with IgE myeloma and into normal individuals the half-life of free IgE molecules was estimated to be 2.7 days (Waldmann et al., 1976). However, large amounts of this class of antibodies are tightly bound to FcεRI-bearing cells (Kinet, 1999; Kraft and Kinet, 2007; Gould and Sutton, 2008), and also can be captured by the membrane-bound low affinity receptor CD23 (Conrad, 1990; Gould and Sutton, 2008; Acharya et al., 2010) or by the soluble isoforms of FcεRI, CD23 and Galectin-3 (Gould and Sutton, 2008; Dehlink et al., 2011; Platzner et al., 2011), thus free sIgE might be present but unavailable for measurement. The half-life of antigen-specific IgE bound to FcεRI on mast cells and basophils was found to be 56 days in mice, while serum levels of the injected IgE had become undetectable at day 6 (Kubo et al., 2003). In addition, the existence of long-lived IgE memory plasma cells has been demonstrated, suggesting that absolute allergen avoidance may not result in loss of allergy (Mitre and Nutman, 2006; Luger et al., 2009; Luger et al., 2010). Half-life of free isocyanate-specific antibody levels was reported to be 5.8 (TDI), 5.4 (MDI) and 6.7 (HDI) months (Tee et al., 1998), and 3.9 years for TDI respectively (Park et al., 2002). Our patient no. 1 was monitored for 13 years. In year 1, when the patient had been removed from his previous occupational exposure (airborne, dermal) to isocyanates for 5 months, MDI-specific IgE in the range of CAP class 2 was measured and SIC was performed. Nonetheless sIgE peaked in year 3 (CAP class 3) and tIgE was very high in year 5. To note, this patient also showed positive reactions in SPT against 6 common allergens (Table 2), which might have contributed to the tIgE value. 13 years after having been removed from exposure to isocyanates, free sIgE against MDI in the range of the CAP 2 class is still detected. Presumably the patient owns high amounts of IgE producing long-lived plasma cells.

To test our hypothesis that another patient subgrouping may produce more homogenous results in genetic studies, first we used the SIC, specific SPT and specific IgE results to distinguish between challenged patients reacting specifically or not to diisocyanates. The use of total IgE as another indicator is controversial. Usually, tIgE values above 100 IU/mL are considered an indicator for type-I-allergies with regard to common allergens. Sjöstedt et al. (1993) performed a follow-up study with 88 laboratory animal-exposed workers and, besides lung function tests, also analyzed total IgE values. In the groups with laboratory animal allergy (LAA, either manifested as asthma or rhinitis, with positive animal-specific SPT) the tIgE values (Median, U/mL) did not vary over 5 years, with 71 and 52 in the year 1983, and 73 and 45 in 1988. The symptom-free group first had 15, then 21 IU/mL tIgE in serum, while patients with chemical-associated symptoms were found to express 23 and 24 U/mL tIgE in the years 1983 and 1988, respectively. Patients with LAA-rhinitis but negative in the animal-specific SPT had only 10 and 12 U/mL tIgE. Unfortunately, the used IgE method was not specified. Sjöstedt et al. also reported that a positive skin prick test against environmental allergens was associated with LAA asthma. In our studies, 7 of 9 patients of group 1 (SIC/sSPT/sIgE positive, tIgE > 20 IU/mL; Table 2) also showed positive reactions to common allergens in at least 2 SPTs. Tu et al. (2013) analyzed the relationship of total IgE and the diagnosis of allergy in Asian children, and concluded that low total IgE values might help to exclude allergic diseases, while high values are not meaningful.

**Table 4**

Comparison of different patient groups with regard to the GST gene analyses via odds ratios Group 1: SIC and sIgE positive, tIgE > 20 IU/mL; group 2: SIC positive, tIgE < 20 IU/mL; group 3: SIC negative, tIgE > 20 IU/mL; group 4: SIC negative, tIgE < 20 IU/mL. W/W: wild type, W/M: heterozygous mutant, M/M: homozygous mutant.

| Genotype           | Group 1 versus 2 |      |              | Group 3 versus 4 |      |              | Group 1 versus 3  |      |              | Group 2 versus 4  |      |              |
|--------------------|------------------|------|--------------|------------------|------|--------------|-------------------|------|--------------|-------------------|------|--------------|
|                    | SIC pos.         |      |              | SIC neg.         |      |              | tIgE: >20         |      |              | tIgE: <20         |      |              |
|                    | tIgE:<br>>20/<20 | OR   | (95% CI)     | tIgE:<br>>20/<20 | OR   | (95% CI)     | SIC:<br>pos./neg. | OR   | (95% CI)     | SIC:<br>pos./neg. | OR   | (95% CI)     |
| <i>GSTM1</i>       |                  |      |              |                  |      |              |                   |      |              |                   |      |              |
| Present            | 4/3              |      |              | 0/5              |      |              | 4/0               |      |              | 3/5               |      |              |
| Null               | 5/0              | 8.56 | (0.34–213.1) | 6/4              | 15.9 | (0.69–365.4) | 5/6               | 0.09 | (0.00–2.16)  | 0/4               | 0.18 | (0.01–4.35)  |
| <i>GSTT1</i>       |                  |      |              |                  |      |              |                   |      |              |                   |      |              |
| Present            | 5/1              |      |              | 6/7              |      |              | 5/6               |      |              | 1/7               |      |              |
| Null               | 4/2              | 0.40 | (0.03–6.18)  | 0/2              | 0.23 | (0.01–5.74)  | 4/0               | 10.6 | (0.46–244.6) | 2/2               | 7.00 | (0.40–123.4) |
| <i>GSTP1*1105V</i> |                  |      |              |                  |      |              |                   |      |              |                   |      |              |
| W/W                | 3/1              |      |              | 2/6              |      |              | 3/2               |      |              | 1/6               |      |              |
| W/M + M/M          | 6/2              | 1.00 | (0.06–16.0)  | 4/3              | 4.00 | (0.45–35.8)  | 6/4               | 1.00 | (0.11–8.95)  | 2/3               | 4.00 | (0.25–64.0)  |
| <i>GSTP1*A114V</i> |                  |      |              |                  |      |              |                   |      |              |                   |      |              |
| W/W                | 6/3              |      |              | 5/9              |      |              | 6/5               |      |              | 3/9               |      |              |
| W/M + M/M          | 3/0              | 3.77 | (0.15–95.9)  | 1/0              | 5.18 | (0.18–150.6) | 3/1               | 2.50 | (0.19–32.2)  | 0/0               |      |              |

We used the total IgE serum concentration threshold of 20 IU/mL to separate patients into those whose reactions were unlikely to be IgE-mediated from those with possible IgE-mediated reactions. This is in agreement to our finding that none of our patients with total IgE-content less than 20 IU/mL showed any positive reaction in SPT using several common allergens, while 80.0% of all patients with a tIgE-content higher than 20 IU/mL showed positive reactions toward one or more of the tested common allergens. From these results, we deduced the same correlation with regard to isocyanates, reasoning that reactions to isocyanates in SIC combined with a tIgE content less than 20 IU/mL serum would be due to IgE-independent mechanisms. According to these considerations, we decided to group our patients into 4 groups (Tables 1 and 2).

We also measured total IgE at different time points (before as well as 30–60 min and 24 h after challenge). In patients from groups 1 and 3 with initially high values above 100 IU/mL(N) (adjusted to the protein content), we found a decrease of tIgE (Fig. 3). Since the patients from group 3 did not react specifically to isocyanates, this phenomenon is unlikely to be caused by the formation of immune complexes. The specific IgE values also decreased in a non-specific manner, independently from the isocyanate used in the SIC. Since individual IgE molecules have a half-life of 2.7 days and new IgE molecules are freshly generated by long-lived plasma cells there might be another reason for the decrease of IgE (both total and specific) level found in these patients (probably with IgE-mediated immune responses). Perhaps the patients respond with an

**Table 5**

Evaluation of GST analyses according to positive SPT (common allergens) and total IgE values using odds ratios. There is a significant ( $p = 0.01$ ) correlation between *GSTM1* deletions and total IgE values above 20 IU/mL, interpreted as a tendency to develop a type-I allergy. SIC positive individuals had significantly ( $p = 0.08$ ) more often *GSTT1* deletions. The results are also expressed as percentages of mutations related to SIC, SPT or tIgE. Pos. = positive, neg. = negative.

| Genotype           | SIC       |      |             | SPT     |      |              | Total IgE     |            |              |
|--------------------|-----------|------|-------------|---------|------|--------------|---------------|------------|--------------|
|                    | pos./neg. | OR   | (95% CI)    | Yes/No  | OR   | (95% CI)     | >20/<20 IU/mL | OR         | (95% CI)     |
| <i>GSTM1</i>       |           |      |             |         |      |              |               |            |              |
| Present            | 7/5       |      |             | 3/9     |      |              | 4/8           |            |              |
| Null               | 5/10      | 0.36 | (0.07–1.72) | 9/6     | 4.50 | (0.85–23.81) | 11/4          | <b>5.5</b> | (1.05–28.9)  |
| [%]                | [42/67]   |      |             | [75/40] |      |              | [73/33]       |            |              |
| <i>GSTT1</i>       |           |      |             |         |      |              |               |            |              |
| Present            | 6/13      |      |             | 9/10    |      |              | 11/8          |            |              |
| Null               | 6/2       | 6.50 | (1.10–42.1) | 3/5     | 0.67 | (0.12–3.62)  | 4/4           | 0.72       | (0.14–3.82)  |
| [%]                | [50/13]   |      |             | [25/33] |      |              | [27/33]       |            |              |
| <i>GSTP1*1105V</i> |           |      |             |         |      |              |               |            |              |
| W/W                | 4/8       |      |             | 5/7     |      |              | 5/7           |            |              |
| W/M + M/M          | 8/7       | 2.29 | (0.47–11.0) | 7/8     | 1.23 | (0.27–5.67)  | 10/5          | 2.8        | (0.58–13.5)  |
| [%]                | [67/47]   |      |             | [58/53] |      |              | [67/42]       |            |              |
| <i>GSTP1*A114V</i> |           |      |             |         |      |              |               |            |              |
| W/W                | 12/12     |      |             | 9/14    |      |              | 11/12         |            |              |
| W/M + M/M          | 0/3       | 4.67 | (0.42–52.2) | 3/1     | 4.67 | (0.42–52.2)  | 4/0           | 9.8        | (0.47–202.5) |
| [%]                | [0/25]    |      |             | [25/7]  |      |              | [26/0]        |            |              |

increase of cell-bound (Malveaux et al., 1978) or soluble FcεRI-receptors (Dehlink et al., 2011) after the exposure-induced formation of isocyanate-albumin adducts (Sepai et al., 1995; Johannesson et al., 2004), which in turn results in a fast decrease of free IgE levels in the blood.

#### 4.2. Histamine release

The measurement of histamine released after SIC could provide valuable information about the underlying mechanism in SIC positive reactions. Basophils can be used as detector system for the prevalence of bound specific IgE, either in allergen-induced mediator release tests (Demoly et al., 2003) or in flow-cytometry based basophil activation tests assessing the degree of cell activation after antigen exposure (McGowan and Saini, 2013). But all these in vitro assays are hampered by the need of artificial antigens. We visualized the results of histamine analyses for these groups (Fig. 5) and found slight increases in 6 of 9 serum samples taken 30–60 min after finishing SIC. Interestingly, the patients with the highest amount of isocyanate-specific IgE (no. 1) showed no histamine increase. Little is known about the kinetics of histamine release and degradation in humans. Histamine release after degranulation of enriched or purified human basophils after treatment with anti-IgE was reported to be completed after 20 min (Kurimoto et al., 1991; MacGlashan et al., 1994). Of course, the uptake process and the formation of a potentially antigenic isocyanate-adduct might take some time.

To our knowledge, there is also no data available on blood clearance rates of histamine in humans. Experiments with dogs, which were intravenously infused with histamine, showed rapid removal of histamine from the blood: 140 mL blood was cleared of histamine by the kidney in 1 min (Lindell and Schayer, 1958). If the human system behaves comparably, the clearance of 4–6 L blood, contained in one human individual, would take 28–43 min. Since the isocyanate challenge of our patients was performed during a period of 120 min, the maximum histamine release might be reached early during the ongoing exposure, and the histamine clearance might already have started and be nearly finished 60 min after the end of the isocyanate exposure, when the serum sample was taken. It is likely that the patient with the highest amount of detectable free sIgE reacted faster than the other members of group 1, resulting in no more measurable histamine at the sampling time point. Patient no. 7 showed a positive isocyanate-specific SPT and also showed a histamine release 30–60 min after the end of SIC. Patient no. 8 also showed a specific reaction in SPT, but the wheal was smaller than 3 mm in diameter. Nevertheless, this patient released a statistically significant amount of histamine at the same sampling time. The histamine release found in 6 of 9 members of group 1 (positive reactions toward isocyanates, probably IgE-mediated) 30–60 min after finishing the isocyanate challenge supports our decision to classify the patients into 4 groups.

One patient (no. 14) showed no reaction in SIC to isocyanates (and therefore he was added to group 3), but he reacted with an increase of body temperature, from 36.5 to 37.4 °C and 37.0 to 37.4 °C, after exposure to HDI and MDI, respectively. While he showed no histamine release after the first challenge with HDI (Fig. 4) we found a strong histamine release from  $1.68 \pm 0.15$  to  $4.15 \pm 0.49$  ng/mL (normalized to

protein content) 30–60 min after SIC with MDI (not shown). Thus, the measurement of histamine release during (or after) the SIC could provide additional useful information in respect of ongoing cellular reactions induced by isocyanates, although from the clinical point of view the SIC result might be negative. The histamine release result indicates that this patient rather belongs to group 1. There was only one patient in group 4 (= no reaction in SIC to isocyanates, probably no IgE-mediated reactions) that also showed a clear histamine release at the considered time point. This could result from an IgE-independent basophil activation mechanism, i.e. via the complement pathway (Siraganian and Hook, 1976), or a bacterial/viral infection (Norn et al., 1987; Clementsen et al., 1990).

According to our findings, the measurement of histamine release after SIC provides a useful, additional tool to identify individuals reacting in an IgE-dependent manner to isocyanates. But there is a need to identify the time course of histamine release in order to optimize the sampling time point. Since the patients get an indwelling venous cannula to take blood during the challenge, it should provide no additional stress for the patient when more small samples are taken in between the FEV<sub>1</sub> measurements. To our knowledge there is no literature available about in vivo histamine release in the whole blood during/or after the challenge in humans.

#### 4.3. IL-4

While the histamine ELISA assay worked well, and all measured histamine concentrations ranged clearly above the lowest calibrator (0.15 ng/mL; assay sensitivity: 0.02 ng/mL), in our hands none of the two tested IL-4 assays provided reliable results. One assay was calibrated against the new cDNA-derived, recombinant IL-4 standard (NIBSC code: 88/656). We could not detect any natural IL-4 with this assay (sensitivity: <0.27 pg/mL). In contrast, a second assay (sensitivity: < 2 pg/mL) did detect different amounts of IL-4 in our samples, hence the capture antibody of the reputedly more sensitive assay might be raised against the recombinant standard substance and fail to detect natural IL-4. Unfortunately, the results of the second assay were only in the range of the lowest standard (10.24 pg/mL) or below, and the triplicate values fluctuated too much. We tried to enhance the performance of the assay by further dilutions of the standard but this was not successful. Another, commercially available IL-4 assay with calibrators in the range between 31.2 and 200 pg/mL (no. D4050, R&D systems, Minneapolis, USA) was also calibrated using the NIBSC/WHO IL-4 1st International Standard 88/656. The manufacturer of this assay states, that healthy subjects have IL-4 levels below the level of detection (10 pg/mL). Using this assay, mean serum concentrations of 38 pg/mL in healthy control subjects have been reported (Shi et al., 2011). The same provider offers another IL-4 assay (no. HS400, R&D systems), which is also developed on the base of recombinant IL-4. The calibrators are in the range of 0.25–8.0 pg/mL. Sixty serum samples from apparently healthy volunteers were evaluated by the manufacturer for the presence of IL-4. All samples measured less than the lowest IL-4 standard, 0.25 pg/mL. Taken together, IL-4 analysis using common ELISA assays currently is not standardized, and we cannot present reliable results. Nevertheless, IL-4 was shown to be upregulated in human serum of asthma patients compared

to healthy subjects using an assay of R&D (Wu et al., 2014), unfortunately the authors didn't specify the assay method in detail.

In contrast to histamine, the maximum IL-4-release takes much longer, about 5 h for purified human basophils (Clements et al., 1990; Falcone et al., 1996). Therefore, an appropriate later sampling time point presumably would be beneficial for the measurement of IL-4-release after isocyanate provocation, if a suitable, validated IL-4 assay is available.

#### 4.4. Genetic susceptibility

The mutations of glutathione S-transferases genes, coding for antioxidant enzymes involved in detoxification processes (Hayes et al., 2005), are assumed as a susceptibility marker (genetic predisposition to develop asthma). A comprehensive systematic review (Minelli et al., 2010) included meta-analyses of GSTM1 (n = 22 studies) and GSTT1 (n = 19) mutations and found an increased asthma risk associated with the respective null genotype (GSTM1, GSTT1), while the meta-analysis of GSTP1\*1105V (n = 17) suggested a protective role of the Val allele. Unfortunately the heterogeneity of the studies suggests a need for more detailed sub-classification of asthma.

It is proposed that low levels of certain members of the GST family, resulting in a decreased elimination of isocyanates, may lead to elevated formation of isocyanate-albumin conjugates which then act as immunogen (Fisseler-Eckhoff et al., 2011). In contrast a non-enzymatic pathway of MDI reacting with glutathione (GSH) forming MDI-GSH, followed by the transfer

of MDI to human albumin, has been shown (Wisniewski et al., 2013a). Nevertheless, some studies (Table 6) show a relationship of diisocyanate metabolite clearance from serum to urine with GST gene polymorphisms.

There are only few studies available (Table 6) showing the influence of GST mutations on the development of isocyanate asthma. One group found an increased risk for the development of diisocyanate asthma associated with the lack of the GSTM1 gene (Piiirila et al., 2001), while another reported a protective influence of GSTM1 deletion (Yucesoy et al., 2012). On the one hand homozygosity for the GSTP1 105 Val allele was shown to confer protection against TDI-induced asthma and airway hyper responsiveness (Mapp et al., 2002), on the other hand an association of GSTP1\*1105V SNP with diisocyanate asthma was found (Yucesoy et al., 2012). Looking closer at the study data, differences between gene polymorphism distributions can be seen. For instance the control group of Piiirila et al. has a very deviant distribution as compared with the data reported for whole Caucasian populations, while the gene distribution within the asthma case group looks normal (Table 3). In fact, isocyanates might feature different biological activities (Redlich and Karol, 2002), thus genetic polymorphism might have different influences depending on isocyanate exposure, which is often complex in nature. Accordingly, the elimination kinetics in humans after SIC was shown to vary with the used isocyanates (Budnik et al., 2011).

Due to the low patient numbers we could not divide them according to isocyanate exposure. We calculated odds ratios (Tables 4 and 5), comparing the prevalence of GST genotypes in

**Table 6**

Overview of studies addressing the correlation of glutathione S-transferases (GST) polymorphism with diisocyanate asthma (DA) or diisocyanate (D) metabolism. Gray rows indicate studies focusing on isocyanate metabolism, without DA diagnosis. Results of genetic analyses in addition to GSTs are not given. Further information about the GST polymorphism analyzed: GSTP1\*1105V (SNP, exon 5, rs1695/rs947894), GSTP1\*A114V (SNP, exon 6, rs1138272/rs1799811), GSTM1 (null, gene deletion, rs1065411), GSTT1 (null, gene deletion, rs4630), GSTM3 (3 bp deletion, intron 6, rs1799735), GSTA1 (SNP, promotor), GSTP1 (Intron, rs762803), GSTM3 (3'-UTR, rs1109138), GSTM3 (Intron, rs1571858). U: urine, P: plasma. SIC: specific inhalation challenge, BHR: bronchial hyper-responsiveness, PEF: peak expiratory flow, \*all caucasian workers (otherwise not specified). The affiliation of authors to specific working groups (using partially overlapping patient groups/or workers from the same plants) is indicated by [number]. Note the references: Piiirila et al., 2001; Mapp et al., 2002; Littorin et al., 2007; Broberg et al., 2010; Yucesoy et al., 2012; Tinnerberg et al., 2014.

| Reference                       | Question addressed                                                    | Patients [n]                                                                                                         | Diisocyanate asthma (DA) diagnosed by:                                                                                               | Diiso-cyanate         | GST genes (polymorphism)                                                                                   | Result                                                                                                                                                                                                                                                                                                                             |
|---------------------------------|-----------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|-----------------------|------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| (Piiirila et al., (2001) [1])   | Modification of allergic responses to D due to GST genotypes          | 183 exposed workers: 109 with asthma, 73 as controls                                                                 | Occupational exposure, history, PEF, BHR, SIC                                                                                        | HDI, TDI, MDI, (NDI)  | GSTP1*1105V, GSTP1*A114V, GSTM1*0, GSTT1*0, GSTM3 (3 bp del.)                                              | GSTM1*0 genotype had a higher risk for DA, but with a lack of slgE. GSTM3 AA was associated with late reaction in SIC. GSTP1 and GSTT1 polymorphisms showed no correlation regarding DA, atopy (SPT), tlgE, slgE or SIC.                                                                                                           |
| (Mapp et al., (2002) [2])       | GSTP1 as a susceptibility gene for DA                                 | 131 exposed workers: 92 with TDI-induced asthma, 39 asymptomatic subjects*                                           | Occupational exposure, history, BHR, SIC                                                                                             | TDI                   | GSTP1*1105V, GSTP1*A114V                                                                                   | GSTP1 105 Val/Val is protective against DA and airway hyper-responsiveness with increasing effect according to duration of exposure.                                                                                                                                                                                               |
| (Littorin et al., (2008) [3])   | Metabolism of MDI related to GST and NAT polymorphism                 | Follow-up study; year 0: 141, year 2: 158 exposed factory employees* (same plant)                                    | [No diagnosis of DA via SIC. Report of work-related symptoms based on questionnaires (conjunctival-, nasal-, respiratory symptoms)]. | MDI                   | GSTP1*1105V, GSTP1*A114V, GSTM1*0, GSTT1*0, GSTM3 (3 bp del.)                                              | Both GSTP1 105 and GSTP1 114 homozygous wild types showed higher levels of MDA in U than the other genotypes. GSTP1 114 genotype modified the relationship of MDA in P and U. GSTP1 105 Ile/Ile was associated with lower levels of slgG and an increased risk of atopy.                                                           |
| (Broberg et al., (2008) [3])    | Influence of GST, NAT, CYP1A1 and other genes on TDI-related symptoms | 132 exposed workers, 114 non-exposed controls                                                                        | [No diagnosis of DA via SIC. Report of work-related symptoms based on questionnaires (conjunctival-, nasal-, respiratory symptoms)]. | TDI                   | GSTP1*1105V, GSTP1*A114V, GSTM1*0, GSTT1*0, GSTM3 (3 bp del.)                                              | No effects of GST polymorphisms among exposed workers.                                                                                                                                                                                                                                                                             |
| (Broberg et al., (2010) [3])    | Metabolism of TDI related to GST, NAT, CYP1A1 and other genes         | Workers from 11 plants (n = 136), as described by (Littorin et al., (2007))                                          | [No diagnosis of DA].                                                                                                                | TDI, (MDA, IPDI)      | GSTP1*1105V, GSTP1*A114V, GSTM1*0, GSTT1*0, GSTM3 (3 bp del.), GSTA1                                       | GSTP1*1105V shows allele-dosage-effect in P. GSTP1 105 Val/Val resulted in faster elimination of TDA from P to U. GSTM1 and GSTT1 null genotypes significantly changed TDA relationships in P and U. GST polymorphisms are more important for the slow (P → U) than for the fast phase (air → P) of TDI metabolism and elimination |
| (Yucesoy et al., (2012) [4])    | Genetic variants of GST, SOD and EPHX1 related to DA                  | 353 exposed workers, parted in 3 groups*: 1. SIC pos., 2. SIC neg. but symptomatic, 3. SIC neg., but not symptomatic | SIC, occupational exposure, history, respiratory symptoms                                                                            | HDI, MDI, TDI         | GSTP1*1105V, GSTP1 (rs762803), GSTM1*0, GSTT1*0, GSTM3 (3'-UTR, rs1109138), GSTM3*V224I, GSTM3 (rs1571858) | Minor alleles of GSTP1*1105VSNP were significantly associated with protection against DA. GSTP1 rs762803 SNP was associated with DA. GSTM1 deletion conferred protection against DA.                                                                                                                                               |
| (Tinnerberg et al., (2014) [3]) | Metabolism of D related to GST polymorphism                           | 24 exposed workers, samples collected after 2 days of exposure avoidance                                             | [No diagnosis of DA].                                                                                                                | TDI, NDI, MDI, (IPDI) | GSTP1*1105V, GSTM1*0, GSTT1*0                                                                              | Corresponding biomarkers in U and P correlated well for the different D. Polymorphism in GSTM1 genotype seemed to modify the association: nullgenotype → higher metabolite concentration in U.                                                                                                                                     |

our 4 patient groups with regard to SIC reactions and the likelihood of IgE-mediated reactions to isocyanates (as deduced from total IgE values above or below 20 IU/mL), but the low number of individuals in each group masked any strong significance. Mutations of GSTP1\*A114V were rare in our data, in each case coupled to GSTP1\*I105V, and only found in the groups with tIgE > 20 IU/mL (Table 3). There seems to be a higher risk in general for developing IgE-mediated reactions when GSTM1 is deleted, also affecting the risk of developing type I allergic in isocyanate asthma. GSTT1 deletions were found to be significantly higher within our SIC positive than within SIC negative patients.

#### 4.5. Limitations

The low number of tested subjects provides a limitation of this study resulting in a low statistical power. On the other hand, the analyzed patient group with well-controlled laboratory exposure is relatively homogenous, as compared to larger cohorts published.

Some environmental factors (intake of food or medication) may have influenced the individual biomarker, especially histamine levels. Therefore, in forthcoming investigations the intake of histamine-rich food and medication (Baur, 2013b) before the challenge and during the observation period should be strictly avoided and controlled (our patients were instructed to avoid the intake of the respective medicaments 3 days before the SIC test, but no control was possible). The interim period could have influenced both the IgE levels as well as airway reactivity in SIC, leading to some underestimation of isocyanate effects (Pisati et al., 2007).

#### 4.6. Conclusions

Published data suggest that genetic predispositions may play a role in the development of isocyanate asthma (Bernstein, 2011; Baur and Bakehe, 2014; Yucesoy et al., 2014). Assuming that the different pathophysiology underlying disease mechanisms is due to different genetic mechanisms (Moore and Bleeker, 2014), we can postulate that the major prerequisite for such analysis is a reliable discrimination between IgE-mediated occupational asthma and OA from other reasons. Besides skin prick tests (SPTs) and the measurement of specific IgE, the specific inhalation challenges (SICs) are important in clinical diagnosis of allergic asthma (Ortega et al., 2002), but there is a need for further refinement in the sub-classification of various forms of isocyanate asthma.

The subdivision of patients according to their specific reactions toward isocyanates and the tIgE content (below 20 IU/mL any type-I reaction is unlikely) seems to be reasonable in order to discriminate between different genetic mechanisms underlying the development of isocyanate asthma. Although the optimal sampling time has to be further investigated, the analysis of histamine release after the specific challenge test, may disclose patients with IgE-mediated reactions to isocyanates.

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