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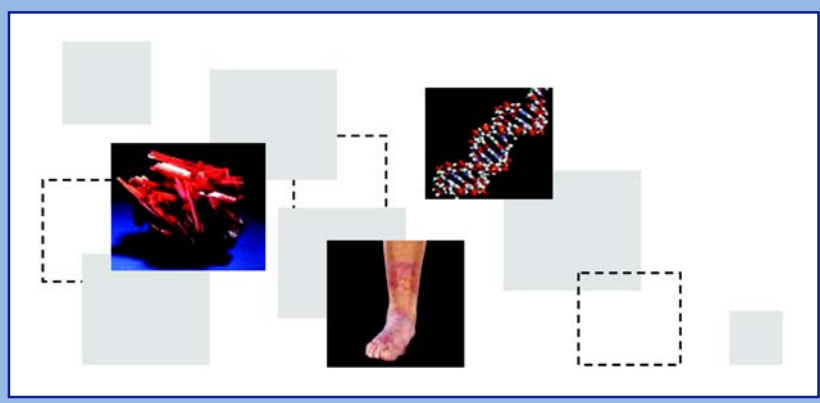
Chromium Allergy

Clinical and Cellular Studies

Malene Barré Pedersen



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Chromium Allergy

Clinical and Cellular Studies

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Malene Barré Pedersen (née Hansen).

ABBREVIATIONS

ACD: Allergic Contact Dermatitis
Cpm: Counts per minute
LC: Langerhans Cell
LTT: Lymphocyte Transformation Test
MET: Minimal Eliciting Threshold
MHC: Major Histocompatibility Complex
PBMC: Peripheral Blood Mononuclear Cells
PHA: Phytohemaglutinin
Ppm: Parts per million
RT PCR: Reverse Transcriptase Polymerase Chain Reaction
SI: Stimulation Index

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1. SUMMARY

1.1 ENGLISH SUMMARY

Chromium salts can cause severe allergic contact dermatitis. Chromium is a transition metal that shows several different oxidation states ranging from –II to +VI. However, only the trivalent Cr(III) and the hexavalent Cr(VI) oxidation states are sufficiently stable to act as haptens. Most studies investigating chromium allergy have been performed with Cr(VI). However, real exposure to chromium from leather products may include both Cr(III) and Cr(VI). The aim of this PhD thesis was to characterise different aspects of allergic contact dermatitis to chromium in previously sensitized patients.

In study I, we performed dose response studies in order to determine the minimum eliciting threshold (MET) concentration for Cr(III) and Cr(VI) in Cr(VI)-sensitive patients. A total of 18 chromium-allergic patients were patch-tested on the back with a dilution series of potassium dichromate (Cr(VI)) and chromium trichloride (Cr(III)). The MET concentration eliciting an allergic reaction in 10% of the patients was calculated from dose response curves to be 0.18 µg Cr(III)/cm^2/48 h (6 ppm Cr(III)) and 0.03 µg Cr(VI)/cm2/48h (1 ppm Cr(VI)). We concluded that although Cr(VI) was confirmed as being the most potent hapten, Cr(III) also demonstrated a significant capacity to elicit allergic reactions at low concentrations. Thus, both Cr(III) and Cr(VI) may play a role in chromium-induced dermatitis.

In study II, we investigated the relation between the content of Cr(VI) and soluble Cr(III) in leather and the ability of the leather to elicit eczema in chromium-allergic patients. A group of 15 chromium-allergic patients with a history of foot dermatitis and leather exposure was exposed to a selection of 14 chromium- and 1 vegetable-tanned leather samples on the upper back. No relation was observed between the measured content of Cr(VI) and soluble Cr(III) in the leather and the elicitation of eczema. Additionally, a prolonged exposure study demonstrated that an extended exposure period might reveal allergenic potential of a leather sample not otherwise identified using an ordinary 48 h-exposure period. We concluded that to evaluate the safety of a leather sample in relation to preventing allergic skin reactions, other, more clinically relevant methods reflecting the actual bioavailable Cr(III) and Cr(VI) fractions should be developed.

In study III we investigated the reactivity to both Cr(VI) and Cr(III) in consecutive patients in order to analyse the clinical pattern in relation to foot eczema and reactivity to Cr(III). Among the 2211 consecutive patients patch-tested, 3.2% had a positive reaction to Cr(VI) of which 44% also had a positive Cr(III) reaction. No Cr(VI) negative patients had a positive reaction to Cr(III). An increased risk for foot dermatitis was found in Cr(VI) positive patients with a concomitant positive or doubtful reaction to Cr(III) compared to Cr(VI) positive patients with no reactions to Cr(III). The increased risk was not due to a higher degree of sensitivity to Cr(VI) but other shoe allergies were more common in the group reacting to both Cr(III) and Cr(VI).

Study IV was a cellular study aiming at finding gene transcripts suitable as *in vit*ro diagnostic markers for allergic contact dermatitis. We used the microarray technology in the identification of differentially expressed genes in allergen-stimulated peripheral blood mononuclear cells (PBMC) from chromium-allergic patients versus healthy controls. A total of 26 genes were differentially expressed by more than two-fold ($p < 0.01$, $q < 9\%$) in allergen-stimulated PBMC from patients compared with controls. Three genes (CASP8, CISH, ETS2) were selected for real-time RT PCR measurements. Analysis of the gene expression in an extended patient/control population indicated that the differential gene expression depended on a proper proliferative response to the allergen *in vitro*. Thus, the three gene transcripts may not provide more information than the traditional proliferative *in vitro* assay on allergic contact dermatitis.

1.2 RESUMÉ

Ph.d. studiet tager udgangspunkt i kontaktallergi overfor kromsalte. Krom kan antage mange oxidationsstadier men kun hexavalent krom (Cr(VI)) og trivalent krom (Cr(III)) er stabile nok til at fungere som haptener. De fleste studier fokuserer på Cr(VI) men eksponering via kromgarvede læderprodukter kan både inkludere Cr(III) og Cr(VI). Formålet med dette ph.d. studie var at karakterisere forskellige aspekter af allergisk kontakteksem over for krom.

Studie I omhandler dosis-respons forsøg med Cr(III) og Cr(VI) til fastlæggelse af grænseværdier for udløsning af allergisk kontakteksem. 18 kromallergiske patienter blev på ryggen eksponeret for fortyndingsrækker af kaliumdikromat (Cr(VI)) og kromtriklorid (Cr(III)). Studiet bekræftede at Cr(VI) var det mest potente hapten. På basis af dosis-respons-kurver blev det beregnet at 1 ppm Cr(VI) (0.03 µg Cr(VI) /cm^2/48 h) vil udløse eksem hos 10% af de kromallergiske patienter. Selvom Cr(VI) var det stærkeste hapten, var Cr(III) langt mere potent end ventet idet den beregnede dosis til elicitering af eksem hos 10% af patienterne var 6 ppm (0.18 µg/cm^{-2}/48 h). Vi konkluderede at både Cr(III) og Cr(VI) potentielt kan spille en rolle i kromallergi.

Studie II havde til formål at belyse sammenhængen mellem mængden af biotilgængeligt Cr(III) og Cr(VI) i kromgarvet læder og læderets evne til at elicitere eksem hos kromallergiske patienter. En gruppe på 15 kromallergiske patienter med nuværende eller tidligere fodeksem samt relevant læderekspONering blev i 48 timer eksponeret på ryggen for 1 vegetabilsk- og 14 kromgarvede læderprøver. Ved aflæsning af eksemreaktionerne var der ingen sammenhæng mellem den målte mængde biotilgængelig Cr(III) og Cr(VI) i læderet og udløsning af eksem. Yderligere viste et 14 dages eksponeringsstudie at 48 timers eksponering ikke vil identificere alle eksemudløsende læderprøver. Vi konkluderede at mere klinisk relevante analysemetoder til måling af biotilgængelig Cr(III) og Cr(VI) i læder er nødvendige for at vurdere risikoen for udløsning eksem hos kromallergiske patienter.

I studie III blev reaktiviteten overfor Cr(III) og Cr(VI) bestemt blandt konsekutive eksempatienter for at vurdere sammenhængen mellem det kliniske billede (fodeksem) og Cr(III) reaktivitet. Blandt de ialt 2211 konsekutive eksempatienter havde 3.2% en positiv lappeprøve for Cr(VI). Blandt disse havde 44% samtidig en positiv lappeprøve for Cr(III). Ingen patienter havde en positiv lappeprøve for Cr(III) uden samtidig også at have en positiv lappeprøve for Cr(VI). Læder var den hyppigst rapporterede eksponeringskilde (54%). Der var en øget risiko for fodeksem blandt Cr(VI) positive patienter med samtidig positiv eller tvivlsom Cr(III) reaktion i forhold til Cr(VI) positive men Cr(III) negative patienter. Den øgede risiko skyldtes ikke en større reaktivitet over for Cr(VI), men der var en større frekvens af andre skoallergier blandt patienter med positiv reaktion for både Cr(III) og Cr(VI).

I studie IV blev gener identificeret, der var specifikt udtrykt i kulturer af kromstimulerede perifert blod mononukleare celler (PBMC) fra kromallergikere i forhold til PBMC fra raske kontroller. Ved brug af Affymetrix Microarrays blev 26 gener identificeret som mindst 2x op- eller nedreguleret ($p < 0.01$, q-value < 9%) i krom-stimulerede PBMC fra patienter sammenlignet med kontroller. Tre gener (CASP8, CISH og ETS2) blev udvalgt til konfirmerende real-time RT PCR målinger. Analyse af en udvidet patient/kontrol gruppe indikerede, at den differentierede genekspression var afhængig af hvorvidt cellerne responderede proliferativt ved tilstedeværelse af krom. De tre udvalgte gener vil dermed ikke bidrage med yderligere diagnostisk information i forhold til det traditionelle proliferationsassay til in vitro diagnostik af allergisk kontakteksem.

2. BACKGROUND

2.1 ALLERGIC CONTACT DERMATITIS

2.1.1 *Clinical Features*

Allergic contact dermatitis (ACD) is an inflammatory skin condition caused by skin contact with sensitizing molecules in the environment. The disease often involves exposed areas on the hands, feet and face. In the acute stage, ACD is characterised by inflammatory processes leading to erythema, swelling, infiltration, papules, vesicles and weeping. In the subacute and chronic stages the lesions become dry, scaly, thick and fissured. Itching is the dominant symptom in ACD (1).

Patients are diagnosed on the basis of physical examination showing clinical characteristics of contact eczema, exposure assessment and a positive epicutanous patch test.

2.1.2 *Sensitizing Molecules, Haptens*

Contact sensitizers or haptens are small molecules with a molecular weight of less than 700 Dalton (2). These haptens penetrate the stratum corneum and arrive at the site of action, the epidermis (3). According to the classical immunology theory, a hapten is too small itself to initiate an immune response (4, 5). In order to obtain sensitizing capacity, the hapten must bind to proteins thereby forming antigenic hapten-protein complexes. In relation to metal haptens, especially Ni^{2+}, two models have been proposed to explain what might be happening at the molecular level (6, 7). The first model proposes that Ni^{2+}-derivatized self-proteins are naturally processed and presented as hapten/peptides by the Langerhans cells (LC) in the skin. The second model proposes a processing independent pathway where Ni^{2+} directly derivatizes the MHC-peptide complexes on the surface of the antigen presenting cells (6–8).

Common sensitizing haptens include metals, biocides/preservatives, fragrance chemicals and dyes.

2.1.3 *Immunological Mechanisms*

The mechanisms of ACD can be separated into two distinct phases: the sensitization phase and the elicitation phase (9). The sensitization phase includes the events following the first contact with the hapten and is complete when the individual is sensitized and capable of giving an ACD reaction. The elicitation phase is initiated upon re-exposure of the same hapten to the skin and results in the clinical manifestations of ACD.

During the sensitization phase, the hapten becomes associated with the peptide-MHC-complex on Langerhans cells situated in the epidermis. The MHC-peptide-hapten complex may be a result of a processed protein-hapten complex recognised by the LC or a direct binding (processing independent) of the hapten to the MHC-peptide complex (6-8). Either way, this activates the LC, initiating a process of cell maturation. Crucial for this maturation is the presence of interleukin-1β (IL-1β) produced by the LC and tumour necrosis factor-α (TNFα) secreted by the local keratinocytes (10;11). The LC matures into a dendritic cell while migrating from the epidermis through the dermis into the afferent lymphatic vessels to the skin draining lymph nodes. Within the paracortical areas of the lymph nodes, the LC encounters naive T cells, which specifically recognise the antigenic peptide-MHC molecule complex. This triggers T cell clonal proliferation and maturation (9). During the maturation process the T cells acquire the cell surface marker, cutanous lymphocyte-associated antigen (CLA), which identifies T cells as skin specific (12). Once CLA is acquired, the T cells are drawn into the circulation.

During the elicitation phase, the effector T cells are recruited into the sites of exposure and this involves complex cell-matrix interactions regulated by adhesion molecule and integrin-receptor interactions with directional guidance supplied by relevant cytokines and chemokines (13). The hapten-protein complex is presented via LC, dendritic cells, macrophages or keratinocytes to antigen-specific memory T cells, which subsequently initiate a cascade of immunological reactions (14). Traditionally, CD4+ T cells were thought to play the predominant role but during the last 15 years evidence has accumulated to suggest that CD8+ cells also play a major role in mediating ACD (15-17). Additionally, both Th1 (e.g. IL-2 and IFNγ) and Th2 (e.g. IL-4, IL-5, IL-10) type cytokines have been demonstrated to take part in the elicitation of ACD even though ACD has traditionally been considered a Th1 response (18-21). Thus, a variety of T cells may contribute to the elicitation and regulation of ACD reactions with the nature of the allergen possibly influencing the relative importance of the functional subsets.

2.2 ALLERGIC CONTACT DERMATITIS TO CHROMIUM

2.2.1 *Chromium - in General*

Discovered in 1797 by the French chemist Louis Nicolas Vauquelin, it was named chromium (Greek chroma: colour) because of the many different colours characteristic of its compounds (22). Chromium is the 24th element on the periodic chart and has an average atomic weight of 52 (Fig. 1).

Chromium is found in nature as chromium ironstone ($FeCr_2O_4$) and as red lead ore ($PbCrO_4$, crocoite) (22–24). The principal and commercially viable ore is chromium ironstone, which is found mainly in southern Africa, Kazakhstan and

Fig. 1. $PbCrO_4$, Crocoite (www.mindat.org/min-1157.html)

the Philippines. On a world wide basis, about 80% of the chromium mined goes into metallurgical applications including stainless steel and others alloys (24). Other applications include the use of chromium in leather tanning, as a wood preservative, colour pigments in paints, laboratory chemicals and accelerators/catalysts (24, 25).

2.2.2 *Chromium as a Hapten*

Chromium is a transition metal that shows several different oxidation states ranging from –II to +VI. However, only the trivalent (Cr(III)) and the hexavalent (Cr(VI)) oxidation states are sufficiently stable to act as haptens (26). The existence of a particular oxidation state depends on various factors such as pH and temperature.

Only in the lower oxidation states (1+ to 3+), are the transition metals present as cations. In the higher oxidation states (4+ to 6+) transition metals are covalently bonded to a non-metal atom, most often oxygen (27). Thus, hexavalent chromium exists as negatively charged oxo-complexes in the form of chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$) ions whereas trivalent chromium exists as the positively charged electrophilic chromic ion (Cr(III)). In proteins, the side-chains of many amino acids contain nucleophilic groups capable of reacting with electrophilic haptens (28). Thus, the degree of electrophilicity determines the degree of protein reactivity, which in turn influences the degree of skin permeability and bioavailability (29). As a consequence, the negatively charged chromate and dichromate ions do not bind the organic substances in the skin whereas the electrophilic chromic ion (Cr(III)) shows a strong affinity for epithelial and dermal tissues forming stable complexes. Therefore, the hexavalent chromium permeates the skin to a larger extent than trivalent chromium since the latter binds to skin proteins thereby becoming captured in the stratum corneum and epidermis. However, Gammelgaard et al.,1992, demonstrated that the lower Cr(III) skin permeation may also be due to a greater skin barrier rejection in that the amount of chromium found in all skin layers was significantly higher when potassium dichromate was applied to the skin compared with both chromium(III) trichloride and chromium(III) nitrate (30). The differences in skin permeability may explain, at least partly, why Cr(VI) compared to Cr(III) is a more potent hapten (see sensitization studies).

As mentioned earlier, haptens must bind to proteins in order to obtain an allergenic capacity. Since the Cr(VI) species does not react with proteins, the oxo-complexes of Cr(VI) are thought to be reduced to Cr(III) within the skin. Thus, Cr(III) is considered to be the actual hapten forming the hapten-protein complex recognised by T cells (31). As a consequence, Cr(VI) may, more correctly, be designated as a pro-hapten.

2.2.3 *Diagnosing Chromium Allergy*

Patients are diagnosed as chromium allergics by using either a patch test concentration of 5000 ppm potassium dichromate corresponding to 1770 ppm elemental Cr(VI) or a clinical equivalent dose of 23 μg/cm2 potassium dichromate (TRUE Test).

Even though the patch test procedure is considered the golden standard in the diagnosis of ACD, it has several disadvantages. Exposing patients to several different contact sensitizers comprises a risk of sensitizing patients to new allergens (32). Also, exposing patients to the allergens to which they are in fact allergic may result in the development of eczema at locations other than the test area. Additionally, the reading of the patch test reaction is subjective depending on the readers knowledge and experience (33).

In vitro assays detecting contact allergies without exposing the patients to the allergens would be of high value. Different test systems including proliferation assays and cytokine detection have been investigated (34–41). However, none has proved capable of replacing the patch test.

2.2.4 *Sensitization Studies*

Predictive skin sensitization assays have been developed in order to identify potential contact haptens. Kligman, 1966, developed a human assay, the maximisation test, which classifies substances into 5 classes according to their allergenic potential (43). Class 1 corresponds to weak haptens and class 5 to extreme haptens. Using this human assay, potassium dichromate was identified as a class 5 hapten and trivalent chromium salts were recognised as grade 3 haptens (43). Today, the sensitization potential of a substance is investigated in animal models only, the two most widely recognised models being the guinea pig maximisation test and the local lymph node assay in mice (44, 45). Both animal models predict potassium dichromate to be a strong hapten (45, 46). With regard to Cr(III), two guinea pig maximisation studies, demonstrated

that the use of Cr(III) gave a lower rate of sensitized animals than when using Cr(VI) (47, 48). Varying degrees of cross-reactions, i.e. inducing with one compound and challenging with another, were reported in both studies.

Van Neer, 1963, demonstrated that when using intradermal application, thereby surpassing the skin barrier, the same degree of contact sensitivity was obtained for trivalent and hexavalent chromium compounds (49). This supports the hypothesis of Gammelgaard et al, that a greater skin barrier rejection of Cr(III) may explain the differences in allergenic potency between Cr(III) and Cr(VI) (30).

2.2.5 *Elicitation Studies*

Threshold concentrations for elicitation of chromium dermatitis have been investigated in several dose response studies. In general, the eliciting threshold concentration of an allergen has been demonstrated to depend on sensitization dose where an increase in the sensitization dose results in a decrease in the elicitation threshold concentration (50). However, the eliciting threshold concentration for a particular patient is typically not a fixed value but varies significantly over time (51).

In Table I, the results from three dose response studies with Cr(VI) (potassium dichromate) and three dose response studies with Cr(III) (chromium trichloride) are presented.

The lowest concentrations eliciting a positive reaction in at least one patient in any of the three studies are between 0.6–1.2 ppm elemental Cr(VI) and 89 ppm Cr(III).

In an article reviewing six Cr(VI) dose response studies, we calculated the minimal eliciting threshold (MET) concentrations for elicitation of eczema in 10% of the participating patients. According to the six Cr(VI) dose response studies, 10% of the chromium-allergic patients will present a positive patch test to a Cr(VI) concentration between 7 ppm and 44 ppm (52). This corresponds to an applied dose between 0.35–0.9 μg/cm^2/48 h. Due to lack of suitable Cr(III) dose response studies, MET concentrations were not calculated for Cr(III) (52). However, looking at Table I, it is clear that in the majority of the patients, much higher concentrations of Cr(III) than Cr(VI) were needed in order to elicit allergic reactions.

2.2.6 *Epidemiology of Chromium Allergy*

In an unselected Danish population of 193 males and 276 females, the prevalence of Cr(VI) allergy in 19 was 0.5% and 0.6%, respectively (calculated from results) (58). In Finland, the prevalence of Cr(VI) sensitivity among 822 human volunteers was found to be 1.7% and in Germany the prevalence in an unselected population of 1141 adults was 1.1% in 1994–1995 (59, 60). In another German study, employing a clinical epidemiology and drug-utilisation research approach, a 9-year (1992-2000) prevalence of Cr(VI) sensitization was determined to be 0.6% (50, 61).

Among consecutive eczema patients in the period 1989–1994, the prevalence of Cr(VI) allergy was 1.8% in Denmark (62). In Germany, the frequency of consecutive patients with a positive reaction to potassium dichromate during the years 1993–1997 declined from 5.8% to 4% for men and remained stable at about 3.6% for women (63).

Table I. *Dose response studies with Cr(VI) (potassium dichromate) and Cr(III) (chromium trichloride). The concentration of Cr(III) and Cr(VI) is given as concentration of elemental Cr. The number and percentage of patients for whom the given concentration of chromium is the lowest eliciting a positive reaction is given for each study. NR: Not reacting to the highest concentration.*

Lowest **Cr(VI)** concentration (ppm) eliciting a positive patch test	Rudzki, 1997 (53)	Allenby, 1983 (54)	Kosann, 1998 (55)	Lowest **Cr(III)** concentration (ppm) eliciting a positive patch test	Rudzki, 1978 (56)	Allenby, 1983 (54)	Fregert, 1964 (57)
	Number of reacting patients *n* (%)				Number of reacting patients *n* (%)		
1770	3 (8%)	3 (21%)		25350			11 (65%)
885	7 (18%)	8 (57%)	4 (33%)	17700			
440			1 (8%)	15581	15 (33%)		
354	6 (16%)			9750			
220			4 (33%)	8850		4 (29%)	
177	8 (21%)			4680			
110			1 (8%)	3100	5 (11%)		
89	9 (24%)	1 (7%)		2340			
35	5 (13%)			1541	1 (2%)		
28			1 (8%)	89		1 (7%)	
9		1 (7%)					
0.6 - 1.2		1 (7%)					
NR			1 (8%)	NR	25 (54%)	9 (64%)	6 (35%)
Total number of participants	38	14	12	Total number of participants	46	14	17

2.2.7 *Exposure to Sensitizing Chromium Compounds*

Occupational exposure to chromium due to contact with cement has been a major cause of chromium dermatitis. The development of chromium allergy is thought to be due to the presence of Cr(VI), formed from a trivalent compound (Cr_2O_3) in the raw material from which cement is produced. The typical patient with allergic cement eczema primarily presents with dermatitis located on the hands, fingers and wrists (64).

In 1979, Fregert et al., suggested a method for reducing the amount of water soluble Cr(VI) in cement by adding ferrous sulphate (65). Since 1981, ferrous sulphate has been added to all cement produced by Aalborg Portland A/S for use in Denmark, thereby reducing the content of Cr(VI) to 2 ppm. The frequency of chromium allergy among construction workers in Denmark has therefore been reduced considerably (64). Now, other Nordic countries have also implemented legislation limiting the Cr(VI) content in cement to below 2 ppm (66). In Germany, the Cr(VI) content has been regulated only recently although not covering all kinds of cement products (67). Thus, cement eczema due to chromium allergy still remains a considerable problem in Germany and in many other countries (67) (68).

As a consequence of the cement regulatory legislation, the epidemiology and the clinical picture of chromium dermatitis in Denmark has changed (62, 69). Leather products have now become the most important known source of chromium exposure in Denmark and chromium allergy has primarily become a consumer problem (62, 69). Leather contains chromium because of the use of basic chromium(III) sulphate for leather preservation - the process of tanning (70). During the tanning process chromium binds to the collagen fibres thereby stabilising the leather. Even though no Cr(VI) is used, small trace amounts of Cr(VI) can occasionally be found in leather articles (71). This is probably due to the oxidation of Cr(III), leading to the formation of Cr(VI) during leather manufacture. Cr(III) may be converted to Cr(VI) by light or heat in the presence of oxidized fats or high pH in the leather. In contrast to Cr(III), Cr(VI) is a poor protein binder and will therefore be more prone to leach out of the leather thereby coming in contact with the skin (72). In addition, despite most Cr(III) being thought to be bound to the collagen fibres in leather, it has been demonstrated that a considerably pool of Cr(III) can be extracted out of the leather (73–75). Thus bioavailable pools of both Cr(III) and Cr(VI) may be present in leather and may potentially take part in both the sensitization and elicitation of leather-induced chromium dermatitis.

3. AIMS OF THE STUDIES

Study I To determine the minimum elicitation threshold concentration for Cr(III) and Cr(VI) in Cr(VI) sensitive patients.

Study II To determine the relation between the content of Cr(III) and Cr(VI) in leather and leather induced elicitation of dermatitis in Cr(VI) positive patients.

Study III To investigate the reactivity to Cr(III) and Cr(VI) in consecutive patients in order to analyse the clinical pattern in relation to foot eczema and reactivity to Cr(III).

Study IV To identify gene transcripts with the potential to function as diagnostic markers for contact allergy to chromium.

4. MATERIALS AND METHODS

All studies were performed after informed consent was obtained from all participants and all studies were approved by the local ethical committee.

4.1 STUDY I

Patients

Eczema patients were included who had previously been patch-tested positive (+: $n = 2$; ++: $n = 16$)) to a diagnostic patch test containing 5000 ppm potassium dichromate ($K_2Cr_2O_7$) (1770 ppm Cr(VI)) at the Department of Dermatology, Gentofte Hospital. All patients had a history of chromium dermatitis, and one had a history of atopic eczema.

Prior to patch testing, none of the participating patients had active dermatitis as they were either free of dermatitis or their dermatitis was in a quiescent phase.

Patch test solutions

Potassium dichromate was used (Sigma-Aldrich, Broendby, Denmark; purity: 99.9%) as a Cr(VI) salt and chromium trichloride ($CrCl_3$) was used as a Cr(III) salt (Sigma-Aldrich, Broendby, Denmark; purity: 99.998%). Serial dilution series were made for patch testing. For Cr(VI), the dilution series ranged from 2 ppm to 1770 ppm elemental Cr(VI) and for Cr(III), the dilution series ranged from 5 ppm to 25,350 ppm elemental Cr(III).

As control, a patch test with pure water was used.

In addition, three solutions containing combinations of Cr(III) and Cr(VI) were included: 5 ppm Cr(VI) and 500 ppm Cr(III), 10 ppm Cr(VI) and 500 ppm Cr(III), 15 ppm Cr(VI) and 500 ppm Cr(III). The different Cr(III) and Cr(VI) concentrations were also applied in separate patch tests. The solutions were made using synthetic sweat (NaCl (5 g/l), lactic acid (1 g/l), urea (1 g/l) and amino acids (1 g/l)). The pH was adjusted to pH 5.5 with ammonia. As control, a patch test with synthetic sweat was used.

Analyses of patch test solutions

The test solutions were chemically analysed for the stability of the oxidation states by Force Technology, Broendby, Denmark. The detection method was a colorimetric assay, in which the Cr(VI) concentration is determined from absorbance at 550 nm by the stoichiometric oxidation products of the diphenylcarbazide reagent. No significant conversion of Cr(III) to Cr(VI) or vice versa was observed in any of the solutions used.

Patch testing

For patch testing, Finn Chambers®) (Epitest, Tuusula, Finland) of 8 mm in diameter were used. The patch tests were applied on the back of the patient with Scanpore® tape (Norgesplaster, Vennesla, Norway). The patches were left for 48 h and readings were done on day 2, 3 and 7. Readings were performed in a non-blinded fashion by the investigator in collaboration with experienced nurses. The reactions were scored according to the ICDRG scale (76) with a 1+ reaction defined as homogenous erythema and infiltration. Further, a more detailed description was made of reactions less prominent than 1+ including erythema only and single follicles. These were defined as very weak allergic reactions.

Data analysis

A two-tailed, non-parametric correlation analysis (Spearman's rank correlation coefficient r_s) was used for the evaluation of *i*) association between the threshold concentrations for Cr(VI) and the threshold concentrations for Cr(III) and *ii*) association between the threshold concentrations of Cr(III) and Cr(VI) for elicitation of a very weak allergic reaction and the threshold concentration for elicitation of a positive reaction.

Prism version 4.0 for Microsoft Windows was used for the construction of the dose response curves for the elicitation of at least a very weak allergic reaction. In order to compare our study with other dose response studies, we also constructed logistic dose response curves for the positive reactions using Excel 1997 for Microsoft Windows.

4.2 STUDY II

Patients

In study II, chromium-allergic patients with a history of foot eczema and suspected leather relevance were included.

Leather analysis - comparing laboratories and analytical methods

An array of leather samples was analysed for the content of Cr(VI) using the available methods: the DIN 53314 and the DS/EN 420 method. The leather analyses were performed by Force Technology, Broendby, Denmark. A fraction of the leather samples were also analysed at the Lederinstitut, Gerberschule, Reutlingen, Germany.

The DIN 53314 method extracts Cr(VI) from 2 g of finely cut-up leather using a potassium hydrogen phosphate buffer at pH 7.7 for 3 h. The extracted Cr(VI) is determined using the diphenylcarbazide colorimetric assay (see study I). The

DS/EN 420 method deviates from the DIN 53314 method mainly in the amount of leather used (10 g) and the extraction time (2 h).

As a reflection of the soluble Cr(III) content, the Cr(III) content in the DIN 53314 buffer was determined using atomic absorption spectrometry.

Exposing patients to leather samples

14 chromium-tanned leather samples and 1 vegetable-tanned control leather sample were selected for 48 h patch testing based on the content of total Cr(VI) and soluble Cr(III) determined by the DIN 53314 method. The leather was applied as small squares (4 cm^2) on the upper back using Scanpore® tape. In addition, one of the leather samples was also selected for a prolonged 14-day exposure study. For the prolonged exposure study, the leather was designed as a bracelet to put around the wrist. Twelve of the 15 patients participated in the prolonged exposure study.

Cr(III) and Cr(VI) solutions

All patients were patch-tested with solutions of Cr(III) and Cr(VI). The concentrations of Cr(III) and Cr(VI) corresponded to the highest concentrations measured in the leather samples used for patch testing.

Test reactions

The clinical reactions were scored according to the scale used in study I.

4.3 STUDY III

Patients

Study III is based on the analysis of consecutive eczema patients, patch-tested at the Department of Dermatology at Gentofte Hospital during the period March 2002–December 2004.

Patch test solutions

All patients were patch-tested with the European standard series including 5000 ppm in petrolatum and a supplemental standard series including 13% chromium trichloride in aqua. The stability of the chromium trichloride solution was analysed as described in Study I.

Patch testing

Patch testing was performed as described in Study I.

Patch test reactions were scored according to the ICDRG scale. The irritative reactions were included in the negative reactions.

Recording of supplemental data

For each patient, the following data were recorded retrospectively: Sex, location of present and past eczema, other contact allergies and presumptive causal exposures.

Statistical analyses

For data management and analysis, the statistical software package SPSS version 12.0 for Windows was used. Risk estimates were expressed as odds ratio (OR) with a 95% confidence interval.

4.4 STUDY IV

Subjects

For the microarray analysis, 3 chromium-allergic patients and 3 non-allergic controls with no history of eczema were included in the study.

For real-time RT PCR analysis, 10 chromium-allergic patients, 1 nickel-allergic patient and 9 non-allergic controls were included.

Cell cultures

Peripheral blood mononuclear cells were isolated from venous blood by density centrifugation on Ficoll-Paque TM PLUS solution (Amersham Biosciences, Hilleroed, Denmark). The mononuclear cells were collected and grown for lymphocyte proliferation tests and gene expression studies.

Cell cultures for lymphocyte proliferation test

PBMC from patients and controls were grown with either phytohemoglutinin (PHA) (Murex Diagnostics Limited, Dartford, England), $CrCl_3$, $NiSO_4$ (Sigma-Aldrich, Broendby, Denmark) or in media alone. Cells stimulated with PHA were grown for 3–4 days and cells stimulated with $CrCl_3$ or $NiSO_4$ were grown for 7 days. T-cell proliferation was estimated by ^{3}H-thymidine incorporation (Amersham Biosciences, Hilleroed, Denmark). The proliferative response was reported as a stimulation index (SI), determined by dividing the counts per minute (cpm) of the stimulated cultures by the cpm of control cultures (PBMC in media alone). A SI value of at least 2 indicates T-cell responsiveness.

Cell cultures for gene expression cultures

Cultures were grown for 24 h with $CrCl_3$, $NiSO_4$ or media alone.

Microarray technology

Total RNA was isolated using the GenEluteTM Mammalian Total RNA Kit (Sigma Aldrich). Generation of cDNA, biotin-

labelled cRNA and HG-U133a GeneChip hybridisation was performed by the RH Microarray Centre at Rigshospitalet, Denmark. The HG-U133a GeneChip comprises more than 22,000 probe sets corresponding to 14,613 different human genes.

Analysis of microarray data

Normalised expression measures from the scanned GeneChips were obtained using the robust multiarray analysis (RMA) procedure (77). Before performing an unpaired t-test comparing patients and controls, the expression measures in the unstimulated cell cultures were subtracted from the Cr(III)-activated cultures to correct for any background gene expression. Subsequently, we calculated *q*-values using the procedure described by Storey (78, 79). The *q*-value gives a measure of the maximal false discovery rate for the significant t-tests at a specified significance level. A q-value less than 10% indicates that more than 90% of the detected differences are likely to be true.

Real-Time RT PCR

Primers for CISH, CASP8 and ETS2 transcripts were planned using the PRIMER3 programme (80). For normalisation of the data, GAPDH was measured.

For real-time RT-PCR, the LightCycler FastStart DNA Masterplus SYBR green I system (Roche Diagnostica, Basel, Switzerland) was applied.

The expression values were log transformed, normalized with GAPDH and corrected for background gene expression before comparing patients and controls using unpaired t-tests.

5. RESULTS AND DISCUSSION

5.1 STUDY I

In study I, 18 patients with a positive diagnostic patch test to 5000 ppm potassium dichromate, were patch-tested with a dilution series of Cr(III) and Cr(VI) in order to determine their minimal eliciting threshold concentration.

The dilution series for Cr(VI) ranged from 2 ppm elemental Cr(VI) to 1770 ppm elemental Cr(VI). The dilution series for Cr(III) ranged from 5 ppm to 25,350 ppm elemental Cr(III).

Patch test reactions to the two dilution series are demonstrated in Fig. 2.

For each patient, the lowest concentration of Cr(III) and Cr(VI) that resulted in *i*) a positive reaction and *ii*) a very weak allergic reaction including erythema only and single follicles, was recorded as the MET concentration (Table II).

None of the patients reacted to the placebo patch test containing pure water. No patients had a positive reaction to the lowest concentrations of Cr(III) and Cr(VI) used. In addition, 7 patients failed to develop a positive reaction to Cr(III), even at the highest Cr(III) concentration of 25,350 ppm. Only in one patient was the positive reaction to the diagnostic patch test concentration of 1770 ppm Cr(VI), corresponding to 5000 ppm potassium dichromate, not reproduced.

In order to elicit a positive reaction in at least one patient, patch tests containing 111 ppm Cr(VI) or 50 ppm Cr(III) were needed. A total of 4 patients had a positive reaction to 111 ppm Cr(VI) and only 1 highly sensitive patient had a positive reaction to 50 ppm Cr(III). The patient with a positive reaction to 50 ppm Cr(III) was also one of the four patients with a Cr(VI) threshold concentration of 111 ppm. In general, the patients with a low Cr(VI) threshold concentration for the elicitation of a positive reaction also had a low threshold concentration for Cr(III) (Fig. 3). The correlation between the threshold concentration of Cr(VI) and the threshold concentration of Cr(III) was significant (Spearman's correlation coefficient, $r_s = 0.62$; $p < 0.01$).

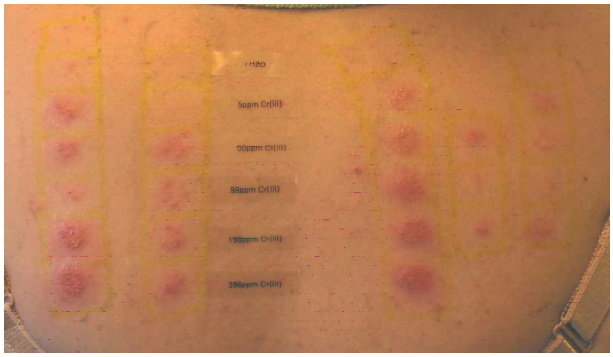

Fig. 2. Patch test reactions to the Cr(III) and Cr(VI) dilution series.

Table II. *The minimal eliciting threshold (MET) concentration was recorded for each patient. The MET is given for both the concentration needed to elicit a positive reaction and a doubtful/follicular reaction.*

Pat.	MET concentration (ppm) for **positive** reactions		MET concentration (ppm) for **very weak allergic*** reactions	
	MET Cr(VI)	MET Cr(III)	MET Cr(VI)	MET Cr(III)
1	111	50	2	50
2	111	198	11	50
3	111	1584	11	50
4	111	3170	11	50
5	221	25350	11	99
6	443	3170	111	50
7	443	25350	2	396
8	443	12675	111	198**
9	443	25350	11	99
10	443	25350	2	99
11	885	-	11	396
12	885	-	11	3170
13	885	-	11	6338
14	885	-	2	1584
15	885	-	111	198**
16	885	-	111	3170
17	1770	6338	221	50
18	-	-	221	12675

* Very weak allergic reactions includes erythema only and single follicles.

** These two patients may have an even lower Cr(III) threshold as they were not tested with concentrations below 198 ppm Cr(III).

Since erythema and single follicles may be regarded as very weak allergic reactions when observed in highly sensitive patients exposed to low allergen concentrations, we recorded these reactions in our dose response study as, in time, these may develop into actual positive reactions (32, 81).

From Table II, it is seen that 4 patients had very weak allergic reactions to the lowest Cr(VI) concentration of 2 ppm. Thus, these patients may in fact react to even lower concentrations of Cr(VI). In contrast, no patients reacted to the lowest Cr(III) concentration of 5 ppm. However, 6 patients reacted to the second lowest Cr(III) concentration of 50 ppm.

There was a significant association between the Cr(III) threshold concentration for the elicitation of a positive

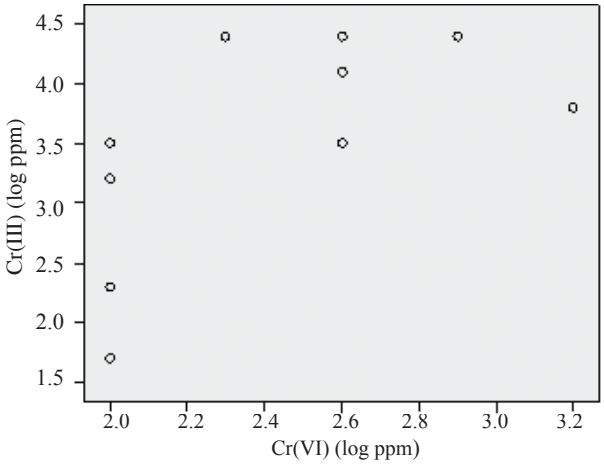

Fig. 3. For each patient, the log concentration of Cr(VI) needed to elicit a positive reaction is plotted against the log concentration of Cr(III) needed to elicit a positive reaction. One circle may represent more than one patient.

reaction and the Cr(III) threshold concentration for the elicitation of a very weak allergic reaction (Spearman's correlation coefficient $r_s = 0.8$, $p < 0.01$). This was also observed for Cr(VI) ($r_s = 0.48$, $p < 0.05$). However, in contrast to threshold concentrations for positive reactions, no correlation was found between the Cr(III) and Cr(VI) threshold concentrations for the elicitation of very weak allergic reactions.

Based on the threshold concentration data for the elicitation of very weak allergic reactions, dose response curves were constructed with the accumulated frequency of patients plotted against the log concentration of Cr(III) and Cr(VI) (Fig. 4).

From the dose response curves, we deduced the Cr(III) and Cr(VI) concentrations resulting in 10% and 50% of the patients having a very weak allergic reaction ($MET_{10\%}$ or $MET_{50\%}$, respectively) (Table III).

The MET concentrations in Table III, demonstrate that, compared to Cr(III), Cr(VI) is the most potent hapten. According to the Cr(VI) dose response curve, 10% of a similar population of patients allergic to potassium dichromate, will react with at least a very weak allergic reaction to a patch test concentration of 1 ppm Cr(VI). In contrast, a concentration of 6 ppm Cr(III) will be needed in order to elicit a reaction in 10% of the patients. Regarding actual positive reactions, 14 ppm Cr(VI) and 188 ppm Cr(III) will elicit a positive reaction in 10% of the patients (dose response curves not shown). The result is in agreement with other Cr(VI) dose response studies finding a $MET_{10\%}$ between 7 and 44 ppm Cr(VI) (52). A more accurately and useful way to present threshold values is in terms of mass of allergens per unit area per time unit (Table III).

In addition to the dose response studies, patch testing with Cr(III) and Cr(VI) separately, we also wanted to investigate whether a combination of Cr(III) and Cr(VI) had a synergistic effect compared to single exposure to Cr(III) and Cr(VI). Patients were patch-tested with three mixed solutions: 5, 10 and 15 ppm Cr(VI) combined with 500 ppm Cr(III) and the corresponding single solutions. The mixed solutions were prepared in synthetic sweat in order to better simulate the exposure situation where Cr(III) and Cr(VI) may be extracted out of leather by patient perspiration. No difference in the response to combined patch tests versus separate patch tests was observed (not shown).

Although numerous dose response studies with Cr(VI) have been carried out, only few also incorporate Cr(III) (54–56, 82–85). In general, Cr(VI) is considered to be the main hapten in relation to chromium allergy and Cr(III) is considered to be of less importance. This is due to the fact that Cr(VI) has been demonstrated to be the stronger hapten of the two. Our study confirms that Cr(VI) is indeed the more potent hapten eliciting a reaction at a concentration as low as 2 ppm. However, Cr(III) also gave rise to allergic reactions at relatively low concentrations. The observation of a highly sensitive patient with a positive reaction to 50 ppm Cr(III) especially underlines the possible significance of Cr(III) in chromium dermatitis. This is in contrast to other dose response studies demonstrating that Cr(III) concentrations well above 1000 ppm are needed to elicit positive patch test reactions. To our knowledge, only one other study demonstrate the allergenic potential of such low Cr(III) concentrations (54).

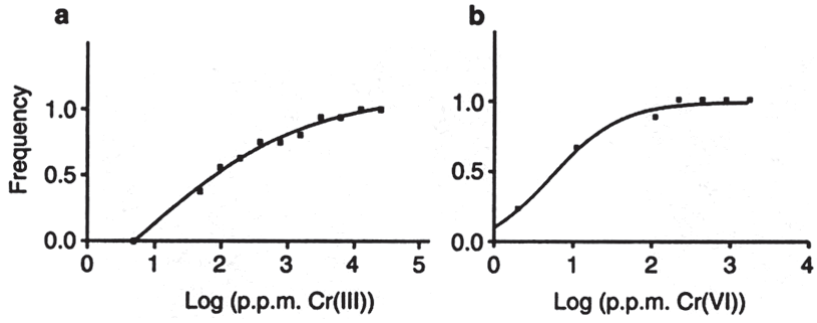

Fig. 4. The accumulated frequency of patients having at least a very weak allergic reaction plotted against the log concentration of chromium.
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Table III. *The minimal eliciting threshold (MET) concentration for Cr(III) and Cr(VI) given as the concentration (ppm) and the amount of Cr(III)/Cr(VI) per unit area per time (µg/cm²/2 days)*
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	Ppm	µg/cm²/2 days
$MET_{50\%}$ for Cr(VI)	5	0.15
$MET_{10\%}$ for Cr(VI)	1*	0.03*
$MET_{50\%}$ for Cr(III)	89	2.7
$MET_{10\%}$ for Cr(III)	6*	0.18*

*These values were below the concentration interval tested or below the lowest concentration to which the patients reacted and should therefore be interpreted with precaution.

Since most Cr(III) dose response studies are relatively old, the difference in Cr(III) sensitivity may partly be explained by changes in exposure pattern over the years. Also, the patients participating in our study are highly sensitive to Cr(VI) with the vast majority having a 2+ reaction to the diagnostic patch test containing 5000 ppm potassium dichromate. Patients having a 2+ reaction compared to a 1+ reaction to the diagnostic patch test are more likely to also react to lower concentrations of Cr(VI) (86). Since we demonstrate that patients reacting to low concentrations of Cr(VI) are also likely to react to low concentrations of Cr(III), this may explain the low Cr(III) threshold concentrations found in this study. Nevertheless, it cannot be ruled out that the observed reactions to the low concentrations are due to the so-called "angry back" phenomena in which strong positive patch test reactions induce reactions in adjacent patch tests (87). However, a dose response study designed to investigate the absence or presence of the "angry back" phenomena concluded that strong reactions to high concentrations of nickel sulphate did not enhance the response to adjacent lower concentrations of nickel sulphate (88).

However, in order to definitely exclude that the strong reactions to the highest concentrations of Cr(VI) and Cr(III) enhanced the reactivity to low patch test concentrations, we should have re-challenged the patients using only the lowest concentrations to which they reacted.

In the majority of dose response studies, only positive reactions are recorded. In this study, we also record very weak allergic reactions, including erythema only and single follicles. Because the participating subjects were all known chromium-allergic patients, single follicles in response to a low allergen concentration was likely to develop into a positive reaction with repeated exposures over time (81). In general, distinguishing a very weak allergic reaction from an irritant reaction on morphological grounds is difficult if not impossible. However, Wahlberg, 2003 demonstrated that in a serial dilution test with nickel sulfate in a nickel-allergic patient, very low concentrations gave rise to only few papules/follicles. In this case, it was concluded that the reaction was indeed relevant because the patient was highly sensitive (32). Since all the participating patients in our study were highly sensitive to Cr(VI), the registration of a few follicles as a very weak allergic reaction to low Cr(VI) concentrations was defined as the most clinically relevant interpretation. In the case of the Cr(III) dilution series, most patients had a minimum 1+ reaction to the highest Cr(III) concentration used and therefore single follicles and erythema in response to the lower concentrations should be regarded as very weak allergic reactions. In contrast, 7 patients did not have a positive reaction to the diagnostic concentration of 25,350 ppm Cr(III), and in these cases it may be more uncertain whether the weak reactions were truely allergic. However, in general these 7 patients had borderline positive reactions with erythema, multiple follicles and infiltration distributed inhomogenous in the test area to the highest Cr(III) concentration followed by gradually decreasing strength of the allergic reactions as the concentration was lowered. In contrast, non-allergic non-eczema controls in general do not react to 25,350 ppm Cr(III) apart from irritative reactions characterised by "silk paper"-skin and discoloration as observed in some of the control subjects participating in study IV (unpublished data). Additionally, false positive reactions with single follicles have been shown mainly to be associated with petrolatum whereas aqueous solutions seem much more stringent and give rise to more false negative reactions (89).

In relation to the mixed solutions containing both Cr(III) and Cr(VI), a reason for not observing a difference between single versus mixed exposure might be that the concentrations used are too high. Since most patients had at least a very weak allergic reaction when exposed to less than 500 ppm Cr(III) as demonstrated by the dose response study, this would "disguise" any subtle differences when combined with small concentrations of Cr(VI). Probably, the concentrations of Cr(VI) should also be lowered to discover any synergistic effect of the combined solutions.

Finally, study I was not designed as a blinded study. Not knowing the location of the different concentrations would have meant that the patch test readings were performed more objectively. Knowing the concentrations and the oxidation state may have affected the patch test scores. However, due to the use of a total of 27 different patch test solutions, we estimated that the risk of mixing up the concentrations would have been too high in a blinded design. In addition, varying the location of the different concentrations would have prevented regional differences in skin reactivity from affecting the results.

In conclusion, although study I confirms that Cr(VI) is the stronger contact sensitizer, it also demonstrates that Cr(III) may elicit allergic reactions at very low concentrations. In fact, the threshold Cr(III) concentration for the elicitation of a positive reaction in at least one patient was lower than for Cr(VI). However, based on the dose response curves for the threshold concentrations for the elicitation of very weak allergic reactions, the $MET_{10\%}$ was found to be 6 ppm for Cr(III) and only 1 ppm for Cr(VI).

5.2 STUDY II

The main known exposure source to chromium in Denmark is chromium-tanned leather (62). Thus, patients presenting with a positive patch test to 5000 ppm potassium dichromate often report a relation between different leather items, especially leather shoes, and the elicitation of eczema. The far most dominant oxidation state present in leather is Cr(III) due to the fact that a Cr(III) compound is used as the tanning agent (70,71). Most of the Cr(III) is bound in the leather and is not considered bioavailable. However, there may be an unbound potentially bioavailable pool of Cr(III) which may leach out of the leather and gain contact with the skin (73–75). In addition, studies have demonstrated the presence of trace amounts of Cr(VI) in leather (71). Thus, the chromium exposure from leather products may comprise both Cr(III) and Cr(VI).

Within the tanning industry, the methods for analysing the pool of Cr(VI) in leather are questioned. It is argued, that the finding of Cr(VI) in the extraction media used for analysing the leather, is not proof of Cr(VI) actually being *in* the leather. The Cr(VI) may have formed during the extraction process (71, 90, 91). In this study, we use two different methods for the determination of the Cr(VI) content in leather. As an expression of the potentially bioavailable pool of Cr(III), we also measure the amount of extractable Cr(III). Finally, we investigate whether a relation exists between the measured amounts of Cr(III) and Cr(VI) in the leather and the elicitation of eczema in chromium-allergic patients.

Leather analysis

A selection of 10 chromium tanned leather samples was analysed for the Cr(VI) content at Force Technology using two different methods: The DS/EN 420 and the DIN 53314 methods (Table IVa). In addition, we also compared two different laboratories (Force Technology, Denmark and Lederinstitut, Reutlingen, Germany) both using the DIN 53314 method (Table IVb).

As seen in Table IVa, the DS/EN 420 method does not detect any measurable Cr(VI). In contrast, the DIN 53314 method finds Cr(VI) above the detection level in 7 of the 10 leather samples. The results from the two different laboratories, both using the DIN 53314 method, were in fairly good agreement (Table IVb).

Since the purpose of study II was to relate the Cr(VI) content to the development of eczema, we chose the DIN 53314 method for the analysis of the leather samples used for patch testing. Whether representing the "true" Cr(VI) content or not, we were only able to rank the leather samples in relation to the Cr(VI) content using the DIN 53314 method. The soluble Cr(III) content was evaluated by measuring the Cr(III) content in the DIN 53314 buffer used for Cr(VI) determination.

For patch testing, 14 chromium-tanned leather samples containing amounts of Cr(VI) and soluble Cr(III) ranging from the lowest to the highest measured were selected for patch testing. As a control, one sample of vegetable-tanned leather was included.

Patch testing with leather samples

Fifteen chromium-allergic patients with past or present foot eczema and suspected leather relevance were patch-tested with the 14 chromium-tanned leather samples and the vegetable-tanned control leather sample (Table V). Five of the 14 chromium-tanned leather samples elicited an allergic reaction in at least one patient and 4 patients reacted to at least one leather sample. The leather sample eliciting a reaction in

Table IV. *Comparison of a) two Cr(VI) determination methods: the DS/EN 420 and the DIN 53314 method, measured by Force Technology, and b) two laboratories (Force Technology, Denmark and Lederinstitut, Gerberschule, Reutlingen, Germany) measuring the Cr(VI) content (DIN 53314) in the same 4 leather samples.*
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	a		b
Leather sample	Force Tech. DS/EN 420 Mg Cr(VI) /kg (ppm)	Force Tech. DIN 53314 Mg Cr(VI) /kg (ppm)	Lederinstitut, Reutlingen DIN 53314 Mg Cr(VI) /kg (ppm)
1	< 2	4.7	
2	< 2	< 3	
3	< 2	3.0	
4	< 2	4.1	5.6
5	< 2	4.6	4.5
6	< 2	< 3	
7	< 2	7.2	
8	< 2	< 3	< 3
9	< 2	9.2	4.9
10	< 2	4.5	

Table V. *The patients were patch-tested with 14 chromium-tanned leather samples (1–14) and 1 vegetable-tanned control leather (15). Only 5 of the leather samples gave rise to an allergic reaction in at least one patient. Reprinted with permission from Contact Dermatitis (Hansen et al., accepted for publication).*

Leather sample	Cr(III) content mg/kg (ppm)	Cr(VI) content mg/kg (ppm)	Nº of patients reacting
1	12	< 3	3
2	93	< 3	-
3	124	< 3	-
4	139	< 3	-
5	151	< 3	-
6	187	< 3	-
7	200	< 3	-
8	201	< 3	2
9	90	4.1	2
10	156	4.3	-
11	591	4.6	2
12	112	9.2	1
13	157	15.5	-
14*	209	16.9	-
15 (control)	5.8	< 3	-

* The leather sample also used for the prolonged exposure study.

the highest number of patients, was the one with the lowest content of Cr(VI) and soluble Cr(III) (Nº 1 in Table V). The leather sample with the highest concentration of Cr(VI) (16.9 ppm) did not cause any reactions. The leather sample with the highest concentration of soluble Cr(III) (591 ppm) elicited a reaction in 2 patients. In general, no pattern between the measured amount of total Cr(VI) and soluble Cr(III) in the leather and elicitation of eczema was observed. The elicitation of eczema caused by the leather samples could not be explained by other allergies among the participating patients.

In study II, we also included patch tests with aqueous solutions of Cr(III) and Cr(VI) corresponding to the highest concentration measured in the leathers used for patch testing (591 ppm and 16.9 ppm, respectively). We expected that patients reacting to at least one of the leather samples would also react to at least one of the solutions. However, no relation was found between the reactivity to the chromium solutions and reactivity to leather. A total of 5 patients reacted to either 591 ppm Cr(III) or 16.9 ppm Cr(VI) or both. Among these 5 patients, 2 reacted to at least one of the leather samples. Possibly, the lack of any relation between reactivity to solutions and leather is due to the low concentrations used.

Prolonged leather exposure

A total of 12 patients also participated in the prolonged leather exposure study wearing a leather bracelet on the wrist for 14 days. Three patients developed eczema during the 14 days of exposure (Fig. 5). None of the 3 patients reacted to any

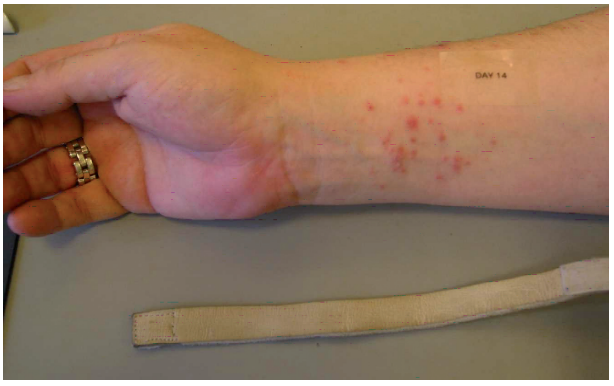

Fig. 5. Elicitation of dermatitis at the wrist due to exposure to a leather bracelet for 14 days.
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of the leather samples in the 48 h exposure study. Also, the leather sample used for the 14 days exposure study did not cause any reactions in any of the 15 patients when exposed on the back for 48 h (Nº 14 in Table V).

Using the DIN 53314 method, no relation was observed between the amount of Cr(VI) and soluble Cr(III) in leather and the elicitation of eczema. However, our study does not reject a connection between the content of Cr(VI) and Cr(III) and the development of eczema. It simply demonstrates that the DIN 53314 method lacks the capacity to determine the relevant bioavailable pools of Cr(VI) and soluble Cr(III) in leather and therefore cannot be used for analysing the Cr(VI) content in relation to avoidance of leather-induced chromium dermatitis. However, it must be underlined that we have no direct evidence that the "chemical agent" eliciting eczema is chromium. Leather contains many different chemicals and in theory, other chemical compounds may cause the observed dermatitis. However, since all the participating patients were chromium allergics with no other obvious allergies with a possible relevance to leather in common, it is highly likely that chromium, whether being Cr(III) or Cr(VI), is the causative agent.

The results from the prolonged exposure study demonstrate that the standard 48 h exposure will not reveal all leathers capable of eliciting eczema: leather shoes and leather watchstraps, in particular, are often worn regularly for long periods and their eczema-eliciting capacities may fail to be detected using the 48 h exposure procedure. Thus a negative patch test to leather does not exclude leather as a relevant chromium exposure source.

5.3 STUDY III

In study III, we investigated the reactivity to Cr(III) and Cr(VI) in consecutive eczema patients in order to analyse the clinical pattern in relation to foot dermatitis and reactivity to Cr(III).

Patch test reactivity

A total of 2211 consecutive eczema patients were patch-tested with 5000 ppm potassium dichromate (1770 ppm Cr(VI)) and 13% chromium trichloride (25,350 ppm Cr(III)) between March 2002 and December 2004.

The reactivity to Cr(III) and Cr(VI) among the 2211 patients is presented in Table VI.

A total of 71 (3.2%) patients had a positive reaction to Cr(VI) of which 31 (44%) also had a positive Cr(III) reaction. Positive reactions to Cr(III) without a concomitant positive reaction to Cr(VI) were not observed.

There was a significant association between the strength of the Cr(VI) reaction (1+ or 2+) and a positive Cr(III) patch test (OR = 8.9; CI: 2.5 -32) (Table VIB). Thus, having a 2+ compared to a 1+ reaction to Cr(VI), significantly increases the risk of a positive reaction to Cr(III).

Among the 71 Cr(VI) positive patients, 49 (69%) were women and 22 (31%) were men. Among the Cr(VI) positive women, 18 (37%) were positive to Cr(III) and among the Cr(VI) positive men, 13 (59%) were positive to Cr(III).

Foot dermatitis and Cr(III) reactivity

A total of 38 (54%) of the Cr(VI) positive patients had past or present foot eczema. Within the group of patients showing a 2+ reaction to Cr(VI), patients having a concomitant positive reaction to Cr(III) had a significantly increased risk of foot dermatitis when compared to the Cr(VI) 2+ positive but Cr(III) negative patients (Table VII). Patients having a 1+ reaction to Cr(VI) and a concomitant positive reaction to Cr(III) had an increased, but non-significant risk of foot dermatitis compared to the Cr(VI) 1+ positive but Cr(III) negative patients.

A significantly increased risk of foot dermatitis was also seen among Cr(VI) 2+ positive patients having a doubtful reaction to Cr(III) compared to the Cr(VI) 2+ positive but Cr(III) negative patients.

Leather exposure

In Cr(VI) positive patients, leather was reported as a relevant exposure source if 1) the patient had a positive patch test using a sample from a suspected leather product and/or 2) the doctor had a suspicion of leather products as causative agents based on the patient history. A total of 38 (54%) of the 71 Cr(VI) positive patients had a suspected leather relevance. Among the Cr(VI) positive patients with a concomitant positive or doubtful reaction to Cr(III), 63% had a suspected leather relevance. In contrast, only 32% of the Cr(VI) positive but Cr(III) negative patients had a suspected leather relevance.

Other contact allergies

Reactions to other shoe-related allergens in patients with foot eczema included rubber chemicals (thiuram mix, carba mix, mercapto mix) and adhesive chemicals (colofonium and p-tert-butylphenol formaldehyde resin). 43% of the Cr(VI)/ Cr(III) positive patients with foot eczema had a positive patch test reaction to at least one non-chromium shoe-allergen. 23% of the Cr(VI) positive foot eczema patients with a concomitant doubtful Cr(III) reaction had reactions to other shoe allergens. In the Cr(VI) positive but Cr(III) negative foot eczema patients, no reactions to other shoe allergens were observed.

The most common other contact allergies among the 71 Cr(VI) positive patients were cobalt chloride (38%) and nickel sulfate (35%). Only 9 of the 71 Cr(VI) allergic patients did not have any other contact allergies. The majority of these (67%) were negative to the Cr(III) patch test.

The overall prevalence of Cr(VI) contact allergy in the 2211 consecutive eczema patients tested between March 2002 and December 2004 was 3.2%. More women (69%) than men (31%) were patch-tested positive to potassium dichromate. Zachariae et al. also found a predominance of women (61%) in the Cr(VI) positive population but the overall prevalence of Cr(VI) allergy was lower (1.7%) compared to our study (62).

Table VI. Reactivity to Cr(VI) and Cr(III) among the 2211 Cr(VI)/ Cr(III) tested patients (A). In (B), the Cr(VI) positive reactions are subdivided into 1+ and 2+ reactions.
[a]Having a 2+ reaction compared to a 1+ reaction to Cr(VI), significantly increases the risk of a positive reaction to Cr(III) (OR: 8.9; CI: 2.5–32). Reprinted with permission from Contact Dermatitis (Hansen et al., 2006).

A

		Cr(III)			
		1+/2+	+?	Neg	**Total**
Cr(VI)	1+/2+	31	18	22	**71**
	+?	0	22	194	**216**
	Neg	0	26	1898	**1924**
	Total	**31**	**66**	**2114**	**2211**

B

		Cr(III)			
		1+/2+	Neg	**Total**	
Cr(VI)	1+	6	15	**21**	OR=1
	2+	25	7	**32**	[a]OR=8.9 (CI: 2.5–32)
	1+/2+	31	22	**53**	

Table VII. *Odds ratio (OR) for foot dermatitis in Cr(VI) 1+ patients with :*
[a]1+/2+ reaction or [b]doubtful reaction to Cr(III) compared to [c]Cr(VI) 1+ patients with no reaction to Cr(III).
OR for foot dermatitis in Cr(VI) 2+ patients with : [d]1+/2+ reaction or [e]doubtful reaction to Cr(III) compared to[f]Cr(VI) 2+ patients with no reaction to Cr(III).
OR for foot dermatitis in Cr(VI) 1+/2+ patients with : [g]1+/2+ reaction or [h]doubtful reaction to Cr(III) compared to [i]Cr(VI) 1+/2+ patients with no reaction to Cr(III).
Reprinted with permission from Contact Dermatitis.
(Hansen et al., 2006)

	Foot eczema %(*n*/total)	OR (95% CI)
Subgroups of Cr(VI) reactions		
Cr(VI) : 1+; Cr(III):1+/2+ (n=6)	50 (3/6)	2.0[a] (0.3-13.7)
Cr(VI): 1+; Cr(III): +? (n=10)	50 (5/10)	2[b] (0.4 - 10.3)
Cr(VI): 1+; Cr(III): N (n=15)	33 (5/15)	1[c]
Cr(VI): 2+; Cr(III): 1+/2+ (n=25)	72 (18/25)	**12.8**[d] **(1.3 - 125)**
Cr(VI): 2+; Cr(III): +? (n= 8)	75 (6/8)	**18**[e] **(1.3 - 256)**
Cr(VI): 2+ ; Cr(III): N (n= 7)	14 (1/7)	1[f]
Pooled groups of Cr(VI) reactions		
Cr(VI): 1+/2+; Cr(III): 1+/2+ (n=31)	68 (21/31)	**5.6**[g] **(1.7 - 18.7)**
Cr(VI): 1+/2+, Cr(III): +? (n=18)	61 (11/18)	**4.2**[h] **(1.1-15.9)**
Cr(VI): 1+/2+; Cr(III): N (n=22)	27 (6/22)	1[i]

Traditionally, men have been in the majority among the Cr(VI) positive patients due to the occupational exposure to cement containing Cr(VI) (92). However, since the introduction of chromate regulation in 1981 allowing a maximum of 2 ppm Cr(VI) in cement, the epidemiology of chromium dermatitis in Denmark has changed (69). In agreement with the study by Zachariae et al, the present study, found that the most frequently suspected cause of chromium dermatitis is leather products (62). The fraction of patients reporting leather relevance was estimated to be 54% of the Cr(VI) positive patients. However, it must be underlined, that leather relevance is rather an imprecise parameter since the recorded leather relevance was often based only on a doctors suspicion based on clinical examination and the history of the patient. Far from all patients with suspected leather relevance were patch-tested with the suspected leather item. Additionally, a negative leather patch test would not exclude leather possibly being the causative agent as demonstrated by the prolonged leather exposure experiment in study II. Finally, when medical staff record anamnestic information, the registration of leather exposure might be rather biased in that leather relevance may be investigated more thoroughly in patients positive to both Cr(VI) and Cr(III) compared to patients only positive to Cr(VI). Since leather shoes are a frequent cause of dermatitis in chromium-allergic patients (62), foot eczema may be regarded as a more objective indication of leather-induced chromium dermatitis, even though other shoe-relevant allergens may be important confounding factors.

An increased risk for foot dermatitis was found in Cr(VI) positive patients with a concomitant positive or doubtful reaction to Cr(III) compared to Cr(VI) positive patients with no reactions to Cr(III). The increased risk was not due to a higher degree of sensitivity to Cr(VI) since it was observed within the group of patients having a 2+ reaction to Cr(VI). However, we cannot exclude that the 2+ patients with foot eczema may have lower minimal eliciting threshold concentrations to Cr(VI) compared to the 2+ patients with no foot eczema. Compared to patients positive only to Cr(VI), patients also having a positive reaction to Cr(III) often also had positive reactions to other shoe allergens. Thus, the increased risk for foot dermatitis in the patients positive to both Cr(III) and Cr(VI) may be caused by shoe allergens other than chromium. The link between Cr(III) and other shoe-related allergens is unknown. One may guess that a primary Cr(III) and Cr(VI) allergy resulting in foot eczema will cause a poor skin barrier thereby promoting development of other shoe allergies. This could also work the other way around, where the presence of other shoe allergies would increase the risk of developing Cr(III) sensitivity. Another possibility could be that the high susceptibility to develop multiple shoe allergies may be caused by patient related factors.

5.4 STUDY IV

The standard procedure for diagnosing ACD is patch testing. However, this method has several disadvantages, including risk for patient sensitization and subjective interpretation (32). The development of a new *in vitro* test system would therefore be valuable. Proliferation assays have been evaluated as such *in vitro* assays for identifying and diagnosing patients with ACD (34, 38–40). However, the specificity and sensitivity of these *in vitro* assays have not been satisfactory. Depending on the allergen in question, a significant number of patients fail to respond proliferatively *in vitro*. In addition, nickel-reactive cells have been demonstrated to be present in patch test negative individuals with no history of allergic disease (93).

In study IV, we investigated whether gene transcripts were more suitable markers for the distinction between allergics and non-allergics. First, we used the microarray technology in the identification of differentially expressed genes in allergen-stimulated peripheral blood mononuclear cells (PBMC) from chromium-allergic patients versus healthy controls. Second, we investigated the usefulness of selected genes for the identification of patients versus controls in expanded patient/control populations.

Lymphocyte proliferation test

When T cells are presented to their specific allergen, they start to proliferate (clonal expansion) (94). To determine whether *i*) the PBMC from the chromium-allergic patients respond proliferatively to the presence of chromium, *ii*) the PBMC from the controls have no response to the presence of chromium and *iii*) PBMC from both patients and controls respond to stimulation with a positive control in the form of the mitogen phytohemaglutinin (PHA), we performed the lymphocyte transformation test (LTT). The LTT assay measures the degree of cell proliferation by the incorporation of radioactive labelled thymidine into the DNA string. The degree of chromium- and PHA-stimulated DNA synthesis in PBMC obtained from chromium allergics and corresponding controls is shown in Table VIII.

Among the chromium allergic patients, the proliferative response to Cr(III) varied from very weak (SI = 1.5) to very strong (SI = 281). None of the controls was stimulated to proliferate in the presence of Cr(III). Both patients and controls reacted proliferatively to stimulation with PHA, indicating that all cell cultures were viable and capable of proliferating *in vitro*. The high degree of variation in the proliferative response to PHA was due to differences in the incubation time (3 or 4 days) and a high degree of variation in the background proliferation in the unstimulated cell cultures (if the spontaneous proliferation is high, it may be more difficult to increase the SI by adding antigen) (95).

The three most chromium-responsive PBMC cultures among the patients (P1, P2 and P5) and three corresponding controls (C1, C2 and C3) were selected for gene expression analysis using the microarray technology.

The microarray study

A total of 26 genes exhibited a more than two-fold difference in expression in Cr(III)-activated PBMC from patients compared to controls ($P< 0.01$; $q < 9\%$) (Table IX).

In order to validate the expression data obtained using the microarray technology, we analysed the expression of three selected genes using real-time RT PCR in an extended patient/control population including 4 additional patients (P3, P4, P6 and P7 in Table VIII) and 1 additional control (C4 in Table VIII).

According to the microarray analysis, two of the genes, CISH and ETS2 were upregulated and the third gene, CASP8, was downregulated in the patients compared with the controls (Table IX). Using real-time RT PCR, CASP8 demonstrated a significant ($p = 0.02$) reduction in the mean expression level in the allergic patients compared with the controls subjects (Fig. 6). A non-significant increase in the mean expression for the ETS2 and CISH was found in allergic patients compared with control subjects. The gene expression seemed to correlate with the degree of proliferation (correlation coefficient: CISH: $r = 0.94$; $p = 0.056$; ETS2: $r = 0.69$; $P = 0.31$) (Fig. 6). Accordingly, if only the PBMC from patients with a high proliferative response (the three patients participating in the microarray study and the additional patient with SI =8), the mean expression level for ETS2 and CISH is significantly ($p < 0.05$) increased in patients compared with controls (Fig. 6).

To investigate more thoroughly whether the gene expression between non-proliferating PBMC from patients and controls could be differentiated, we analysed the expression of the three genes at different time points of stimulation. For these temporal studies, PBMC cultures from 6 chromium-allergic patients and 6 corresponding controls were included. None of the PBMC cultures from the patients had responded to the presence of Cr(III) measured by the LTT assay. The PBMC cultures were stimulated with Cr(III) for 0 h, 1 h, 8 h, 24 h and 48 h and the expression of CISH, ETS2 and CASP8 was analysed using real-time RT PCR.

At no time point, was the expression significantly different for patients compared to controls for any of the three genes. However, CISH exhibited a "near significant" differential expression between patients and controls at the 24 h time point ($p = 0.07$) (Fig. 7).

To determine whether the expression profile was valid for patients allergic to a non-chromium contact sensitizer, a nickel-allergic patient and two corresponding controls were included in the study. The PBMC from the nickel-allergic patient responded strongly to the stimulation with nickel sulphate according to the LTT protocol (SI = 22). Interestingly, the up-regulation of ETS2 and CISH and the downregulation of CASP8 was also observed in the nickel-stimulated nickel-allergic patient compared with the 2 controls (Fig. 8).

Many studies have been published, regarding the development of *in vitro* diagnostic tests for ACD (34–41). The studies use proliferation assays or cytokine expression in the distinction between PBMC from sensitized versus non-sensitized individuals. However, the specificity and sensitivity of the *in vitr*o tests vary greatly depending on the allergen

Table VIII. *Cr(III)- and PHA-induced lymphocyte DNA synthesis in PBMC cultures. The subjects indicated in bold were included in the microarray study. Patients: P1 - P7; Controls: C1-C4. ND = Not done. SI: mean counts per minute (cpm) in stimulated cells divided by the mean cpm in unstimulated cells. Reprinted with permission from Contact Dermatitis. (Hansen et al., 2005).*

	P1	**P2**	P3	P4	**P5**	P6	P7	**C1**	**C2**	**C3**	C4
SI (Cr(III) - stimulated cells)	281	12	1.5	4	28	2	8	1	1	1	1
SI (PHA-stimulated cells)	6	352	4	22	44	2	ND	6	3	7	3

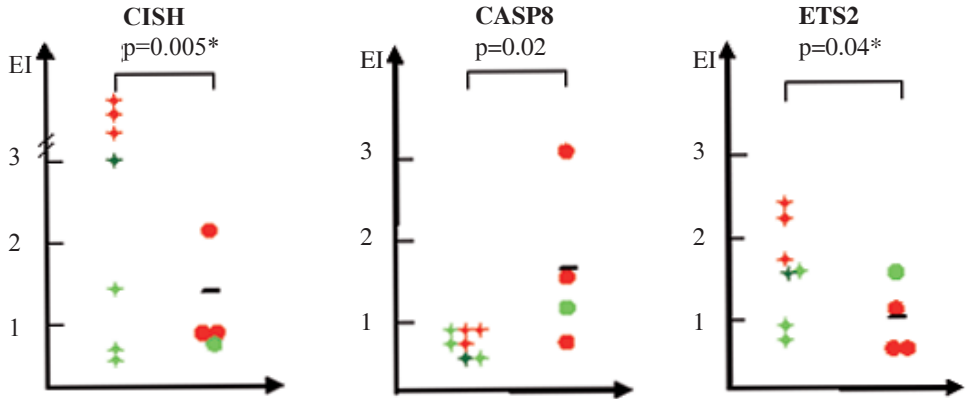

Fig. 6. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) of CISH, CASP8 and ETS2 for i) the 3 patients (red stars) and the 3 controls (red circles) from the microarray study and ii) 4 additional patients (green stars) and one additional control (green circle). The additional patient with the highest SI value (SI = 8) is indicated by a dark-green star. The mean of all four controls is indicated (-).
EI: Expression Index (mRNA level in Cr(III)-stimulated cell cultures divided by mRNA level in unstimulated cells cultures).
CASP8 was significantly downregulated when comparing all 7 chromium-allergic patients to the 4 controls (p = 0.02).
* For CISH and ETS2, the differential expression between the patient and control group was significant only when excluding the three patients with a low-proliferative response.

used, and the usefulness of these tests remains to be evaluated. Studies investigating chromium find that around 70% of the chromium allergic patients respond proliferatively *in vitro* (34, 39). In order to increase the *in vitro* detection of allergic patients without increasing the number of false positives, different approaches have been tried to optimise the detection methods. These approaches involve supplementation with different cytokines (38).

In study IV, we investigated whether the analysis of thousands of transcrips would identify sensitive markers for contact allergy. Using the microarray technology, we detected differentially expressed genes in allergen-stimulated PBMC from patients compared with controls. The analysis of three selected genes showed that the differential gene expression was reproducible in proliferating PBMC from patients compared to non-proliferating controls. In addition, the gene expression changes were also valid for a nickel-allergic compared to corresponding controls. However, the real-time RT PCR analyses did not reveal enhanced segregation between patients and controls when including patients not reacting proliferatively to the presence of chromium. Since the expression profile of three selected genes was reproduced only in proliferating patients, the gene transcripts would not be superior to the LTT assay. Thus, these genes may themselves not be better than the proliferation assay at identifying allergics from non-allergics

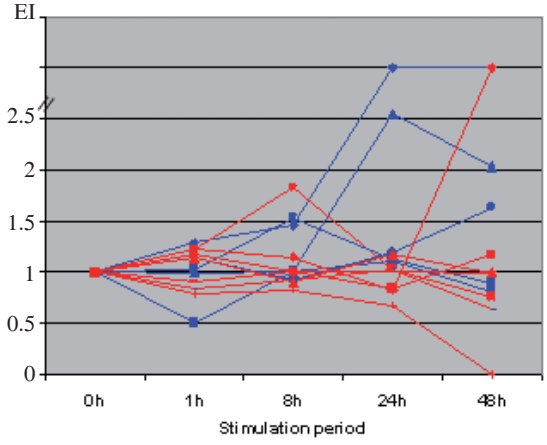

Fig. 7. Expression of CISH after 0 h, 1 h, 8 h, 24 h and 48 h Cr(III) stimulation in patients (blue) and controls (red). After 24 h of stimulation, the biggest difference between patients and controls was observed. The highest expression values are fitted to the diagram.
EI: Expression index.

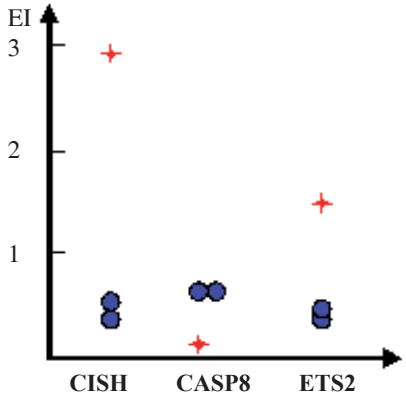

Fig. 8. Real-time RT PCR analyses of CISH, CAPS8 and ETS2 in nickel-stimulated PBMC from 1 nickel-allergic patient (red star) and 2 controls (blue circle). Reprinted with permission from Contact Dermatitis. (Hansen et al. 2005).
EI: Expression Index.

Table IX. *26 genes were identified as being differentially expressed ($p < 0.01$; $q < 9\%$) in allergen-actived PBMC from chromium-allergic patients compared with healthy controls. The genes have a fold change equal to or higher than 2. The genes are segregated into 5 functional groups according to the Gene Ontology Consortium (http://geneontology.org). Reprinted with permission from Contact Dermatitis. (Hansen et al., 2005).*

GenBank Accession No	Gene name	Gene Symbol	Direction of regulation	Fold Change
Immune response/ inflammatory response				
NM_007115	Tumour necrosis factor, alpha-induced protein 6	TNFAIP6	↑	3.64
NM_005849	Immunoglobulin superfamily, member 6	IGSF6	↑	2.09
NM_000417	Interleukin 2 receptor, alpha	IL2RA	↑	2.03
NM_001465	FYN binding protein (FYB-120/130)	FYB	↓	-2.10
NM_016562	Toll-like receptor 7	TLR7	↓	-2.30
NM_000570	Fc fragment of IgG, low affinity IIIb, receptor for (CD16)	FCGR3B	↓	-3.60
Cell Growth/ maintenance				
NM_002438	Mannose receptor, C type 1	MRC1	↑	4.21
NM_002648	Pim-1 oncogene	PIM1	↑	2.97
NM_005239	**v-ets erythroblastosis virus E26 oncogene homologue 2**	**ETS2**	↑	**2.23**
NM_005239	v-ets erythroblastosis virus E26 oncogene homologue 2	ETS2	↑	2.18
NM_006185	Nuclear mitotic apparatus protein 1	NUMA1	↓	-2.80
Apoptosis				
NM_000657	B-cell CLLlymphoma 2	BCL2	↑	2.23
NM_001228	**Caspase 8, apoptosis-related cysteine protease**	**CASP8**	↓	**-2.10**
Metabolism and synthesis				
NM_005525	Hydroxysteroid (11-beta) dehydrogenase 1	HSD11B1	↑	3.03
NM_003364	Uridine phosphorylase 1	UPP1	↑	2.92
NM_004527	Mesenchyme homeo box 1	MEOX1	↑	2.91
NM_012413	Glutaminyl-peptide cyclotransferase (glutaminyl cyclase)	QPCT	↑	2.88
NM_003679	Kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	KMO	↑	2.80
NM_006406	Peroxiredoxin 4	PRDX4	↑	2.00
NM_003032	Sialyltransferase 1	SIAT1	↓	-2.00
NM_025080	Asparaginase-like 1	ASRGL 1	↓	-2.40
Cell Communication / signal transduction				
NM_145071	**Cytokine inducible SH2-containing protein**	**CISH**	↑	**4.20**
NM_003877	Suppressor of cytokine signalling 2	SOCS2	↑	3.80
NM_000632	Integrin, alpha M, CD11b	ITGAM	↑	2.45
NM_003149	src homology three (SH3) and cysteine rich domain	STAC	↑	2.22
NM_003745	Suppressor of cytokine signalling 1	SOCS1	↑	2.13
NM_001619	Adrenergic, beta, receptor kinase 1	ADRBK1	↓	-2.10

even though CISH revealed a tendency towards upregulation in PBMC from non-proliferating patients compared with controls. Nevertheless, although the expression of individual genes may not provide an entirely consistent outcome, the combination of expression changes for numerous candidate genes may enable reliable discrimination between allergics and non-allergics. Additionally, among the 26 differentially expressed genes identified with the microarray technology, there may be some, which may provide a more sensitive differentiation between patients and controls. All 26 differentially expressed genes should be analysed in order to identify the most sensitive.

The gene changes identified in this study may be due to either allergen-specific transcription or the general processes activated in relation to increased lymphocyte turnover. Thus, these gene changes might just as well have been observed in PHA-stimulated cells. However, both unspecific and allergen-specific induced gene changes would be valuable provided they were capable of distinguishing between PBMC from patients and controls.

Possibly the gene transcripts could offer a higher degree of sensitivity and specificity, if they originated from cell cultures comprising only a subpopulation of PBMC. It has been demonstrated that antigen-induced gene expression profiles in PBMC cultures do not reflect those of T-lymphocyte subsets (96). Thus, specific allergen-induced gene transcripts changes in the specific T cells may be overlooked upon examination of the entire PBMC population. The gene expression changes related directly to the downstream signalling events after specific activation of the T-cell receptor might provide the most sensitive signals. However, in the mixed cell populations, these signals may not be identified.

It could also be argued that the lack of differential gene expression in non-proliferating patients versus controls is unsurprising since the list of genes identified using the microarray technology is based on the analysis of the gene expression in highly proliferating PBMC from patients versus that in non-proliferating controls. Thus, performing a similar microarray experiment using non-proliferating PBMC from patients could possibly identify more sensitive markers. This will be successful only if there is a subtle stimulation of the specific T cells not detectable using the proliferation assay. If the non-proliferating PBMC cultures simply reflect the total absence of any effect of the presence of specific allergen this would be pointless.

If a diagnostic test should have any usefulness, the optimal *in vitro* conditions for each of the vast array of contact sensitizers from the standard series should be investigated. To our knowledge, the research concerning *in vitro* diagnostics has mainly focused on nickel, chromium and to some degree isothiazolinones (34–36, 39, 40, 97). Furthermore, patient-related factors influencing the outcome of an in vitro test should be identified, e.g., the *in vitro* response of PBMC to nickel stimulation has been demonstrated to be influenced by the atopic status of the patient (98). Finally, the *in vitro* test only detects sensitization but does not predict whether the sensitization will lead to any clinical symptoms. Thus, the relation between the *in vitro* and the *in vivo* response should be established.

In this study, we use the microarray technology for the analysis of gene transcripts for use in *in vitro* diagnostic tests for ACD. The microarray technology may also be employed to mechanistic studies in order to gain insight into the pathogenesis of the skin disease. Analysing the gene transcripts in skin biopsies from different time points during the development of ACD would possibly give valuable information leading to increased understanding of the disease and possibly identify new therapeutic targets.

6. GENERAL DISCUSSION AND PERSPECTIVES

The only way to avoid ACD is to avoid the allergen in question. However, no matter how good the intentions may be, this can be difficult and can put unacceptable limits on life. Instead of the responsibility being put on the patient, appropriate legislation should be implemented, securing that the majority of the patients will not be exposed to allergen concentrations equal to or above the eliciting thresholds.

Regulations that have a preventive effect on the development of ACD have been implemented for different allergens including nickel and chromium (99;100). Legislation stating that the content of Cr(VI) in dry cement must not exceed 2 mg per kg (2 ppm) was passed in Denmark in 1983 (Arbejdstilsynets bekendtgørelse 1983) (92). However, already in 1981, Aalborg Portland A/S, the only manufacturer of cement in Denmark, patented a method whereby the amount of chromate in cement could be reduced (92). The method was originally suggested by Fregert et al. 1979 (65). The regulation led to a significant decline in the prevalence of chromium allergy among workers in the construction industry in Denmark (99). Other Nordic countries have also implemented legislation limiting the Cr(VI) content in cement to below 2 ppm (66).

Leather contains chromium, that is Cr(III), due to its use as a tanning agent (70). Even though no Cr(VI) is used in the tanning process, Cr(VI) has been detected in Cr(III)-tanned leathers (71). The presence of Cr(VI) is thought to be due to the oxidation of Cr(III) during the tanning process. However the leather industry argues that the detection of Cr(VI) is an artefact caused by the analytical method itself.

In contrast to Cr(III), Cr(VI) does not bind to the collagen fibres and does not have any function within the leather.

Study I demonstrated that not only Cr(VI) but also Cr(III) may elicit ACD in chromium-allergic patients. In study III, an increased risk for foot dermatitis in patients with a positive patch test to both Cr(III) and Cr(VI) compared to patients positive to only Cr(VI) indicated a role of Cr(III) in foot dermatitis and thereby leather-induced chromium dermatitis. Since Cr(VI) has no use in leather, the regulation should aim at preventing Cr(VI) formation. In contrast, the regulation should not put limits on the total content of Cr(III) but only on the *release* of Cr(III) from leather. A leather sample with a high content of Cr(III) will be safe for use providing the Cr(III) is not released from the leather during use. Thus, an appropriate regulation of the content of chromium in leather should state that *i*) no Cr(VI) should be present in the leather and *ii*) the release of Cr(III) should not exceed the eliciting threshold limit for the majority of the chromium-allergic patients.

Threshold levels for the total content of Cr(VI) and the release of Cr(III) from leather products, should be based on appropriate studies simulating the true exposure situation. Study II failed to demonstrate a relation between the measurable amount of Cr(VI) and soluble Cr(III) in leather and its ability to elicit eczema in chromium-allergic patients. Clearly, the methods used for the determination of Cr(VI) and soluble Cr(III) did not reflect the clinically relevant pools of Cr(III) and Cr(VI) in leather. Thus, in order to make an appropriate regulation, relevant analytical methods should be developed.

Whether it is possible to produce chromium-tanned leather that satisfies requirements is unknown. However, specific factors and processing techniques influencing the formation of Cr(VI) during the tanning procedure have been identified. In addition, strategies to reduce the amount of unbound potentially bioavailable Cr(III), including thorough washing procedures, should be implemented. However, not only the newly produced leather should meet the demands: the regulation should also state that the pools of Cr(III) and Cr(VI) should not change in an unfavourable direction during normal usage.

Apart from regulating the Cr(III) and Cr(VI) pools in chromium-tanned leathers, the development of new non-chromium based tanning methods could be a solution. However, the avoidance of leather-induced dermatitis would hardly be a promoter in such a project considering the small number of troubled people. Nevertheless, additional advantages such as a reduction in the amount of problematic waste and the economic and environmental costs involved could possibly be a driving force for the authorities and the leather industry.

7. CONCLUSIONS

Study I The purpose of study I was to determine the minimal eliciting threshold concentrations for Cr(III) and Cr(VI). The Cr(III) and Cr(VI) concentration eliciting an allergic reaction in 10% of the patients was determined to be 0.18 μg Cr(III)/cm^2/48 h (6 ppm Cr(III)) and 0.03 μg Cr(VI)/cm^2/48 h (1 ppm Cr(VI)).

Study II The purpose of study II was to investigate the relation between the measured content of Cr(VI) and soluble Cr(III) in leather and the ability of the leather to elicit eczema in Cr(VI)-allergic patients. No relation was observed using the available methods (DIN 53314 and DS/EN 420) for analysing the content of Cr(VI) and Cr(III).

Study III The purpose of study III was to investigate the reactivity to both Cr(III) and Cr(VI) in consecutive patients in order to analyse the clinical pattern in relation to foot eczema and reactivity to Cr(III). An increased risk for foot dermatitis was found in Cr(VI) positive patients with a concomitant positive or doubtful reaction to Cr(III) compared to Cr(VI) positive patients with no response to Cr(III). The increased risk was not due to a higher degree of reactivity to Cr(VI) but other shoe allergies were more common in the group reacting to both Cr(III) and Cr(VI).

Study IV The purpose of study IV was to identify gene transcripts with the potential to function as diagnostic markers for contact allergy to chromium. A total of 26 genes were identified as differentially expressed in chromium-stimulated PBMC from chromium-allergic patients compared to non-allergic controls. However, analysis of three selected genes indicated that the differential gene expression was related to the degree of proliferation and the identified genes may therefore not provide more information than the traditional proliferative *in vitro* assay on allergic contact dermatitis.

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