UNIVERSITY OF COPENHAGEN
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CONTACT ALLERGY TO METHYLISOTHIAZOLINONE
– OBSERVATIONAL AND EXPERIMENTAL STUDIES

PhD Thesis

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Contact Allergy to Methylisothiazolinone – Observational and Experimental Studies

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This PhD thesis is based on the following manuscripts:

The manuscripts will be referred to by their roman numerals throughout the PhD thesis.

I. Schwensen JF, White IR, Thyssen JP, Menné T, JD Johansen. Failures in risk assessment and risk management for cosmetic preservatives in Europe and the impact on public health. Contact Dermatitis. 2015; 73:133-41.

II. Schwensen JF, Lundov MD, Bossi R, Banerjee P, Giménez-Arnau E, Lepoittevin JP, Lidén C, Uter W, Yazar K, White IR, Johansen JD. Methylisothiazolinone and benzisothiazolinone are widely used in paint: a multicentre study of paints from five European countries. Contact Dermatitis. 2015;72:127-38.

III. Schwensen JF, Bonefeld CM, Zachariae C, Agerbeck C, Petersen TH, Geisler C, Bollmann U, Bester K, Johansen JD. Cross-reactivity between methylisothiazolinone, octylisothiazolinone and benzisothiazolinone using a modified local lymph node assay. Br J Dermatol. 2017;176:176-183.

IV. Schwensen JF, Uter W, Bruze Magnus, Svedman C, Goossens A, Wilkinson M, Giménez Arnau A, Gonçalo M, Andersen KE, Paulsen E, Agner T, Foti C, Aalto-Korte K, Johansen JD. The epidemic of methylisothiazolinone: A European prospective study. Contact Dermatitis. 2016 Dec 28.doi: 10.1111/cod.12733 [Epub ahead of print].

PREFACE

This dissertation is based on scientific work carried out at the National Allergy Research Centre at Copenhagen University Hospital Gentofte, the Department of Immunology and Microbiology at Copenhagen University, and the Department of Environmental Science, Aarhus University from 2014 to 2016.

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Jakob Ferløv Schwensen

ABBREVIATIONS

APC	Antigen presenting cells
BrdU	5-bromo-2'-deoxyuridine
BIT	Benzisothiazolinone
CCET	Cumulative Contact Enhancement Test
CE-DUR	Clinical Epidemiology (CE) and Drug Utilization Research (DUR)
CRA	The Committee for Risk Assessment
DLQI	Dermatology Life Quality Index
EC	The European Commission
ECHA	European Chemicals Agency
EU	The European Union
FDA	American Food and Drug Administration
LC	Langerhans Cells
LLNA	Local Lymph Node Assay
MI	Methylisothiazolinone
MCI/MI	Methylisothiazolinone in 3:1 combination with methylisothiazolinone
MHC	Major histocompatibility complex proteins
OIT	Octylisothiazolinone
ROAT	Repeated open application test
SCC	Scientific and Standardization Committee
SCCS	The Scientific Committee on Consumer Safety
SCCNFP	Scientific Committee on Cosmetic Products and Non-Food Products
SCCP	Scientific Committee on Consumer Products
SI	Stimulation Index
QoL	Quality of Life

SUMMARY

Background and aims

The preservative methylisothiazolinone (MI) is a frequent cause of contact allergy. It is widely used in cosmetic products and its use in leave-on cosmetic products is of particular concern, allegedly contributing to the rapid increase in new cases of MI contact allergy.

Further, MI is added to Danish water-based paint, but no experimental study has hitherto investigated this for paints purchased in the EU. A few epidemiological studies have shown that cross-reactivity between MI and isothiazolinones may exist. However, no study has comprehensively investigated cross-reactivity between MI and common isothiazolinones.

The overall objective of the thesis was to characterize and evaluate the ongoing and unprecedented epidemic of contact allergy to MI. In detail the aims were:

- To retrospectively investigate the epidemiology of MI and preservative contact allergy in a Danish cohort of dermatitis patients over almost three decades.
- To experimentally analyse the content of MI, benzisothiazolinone (BIT) and methylchloroisothiazolinone (MCI) in water-based paint purchased on the European market in five European countries.
- To investigate cross-reactivity between MI, octylisothiazolinone (OIT) and BIT in a modified local lymph node assay (LLNA).
- To prospectively investigate the epidemiology of MI contact allergy in eight European countries and elucidate the exposures regarding products containing MI.

Methods

This thesis is based on four manuscripts. Manuscript I is based on a retrospective cohort of 23 138 dermatitis patients patch tested at Gentofte University Hospital during 1985–2013. Manuscript II builds on data on 71 water-based wall paints randomly purchased at retail outlets in five European countries and quantitatively analysed for content of MI, MCI and BIT. Manuscript III builds on data on immune responses to MI, OIT and BIT in vehicle and MI-sensitised mice and analysed by flow cytometry. Manuscript IV is based on prospectively collected data in eight European countries

collected at 11 centres from 1 May 2015 to 30 October 2015.

Results

Each time a new preservative was introduced on the European market, the overall prevalence of preservative contact allergy in a tertiary hospital cohort increased (Manuscript I). Notably, we found that in former epidemics of preservative contact allergy, the relevance decreased over time, whereas the relevance continued to be high for ongoing epidemics. Further, we showed that MI was found in 93.0% (66/71) of all purchased water-based paint and the concentration ranged from 0.7 to 180.9 ppm (parts per million)(Manuscript II). Interestingly, no difference in the concentration of MI was observed between environmental labelled and non-environmental labelled cans.

In our modified LLNA we showed cross-reactivity between MI, OIT and BIT because the same responses of ear thickness, $CD4^+$ T cells and partly $CD8^+$ T-cells were observed in MI-sensitised mice challenged with MI, OIT or BIT (Manuscript III).

Lastly, we showed that the prevalence of MI contact allergy across eight European countries was 6.0% (205/3434; range 2.6%–13.0%) (Manuscript IV). The dermatitis primarily affected hands (43.4%), face (32.7%), arms (14.6%) and eyelids (11.7%). Relevant MI contact allergy was found in 72.7% (149/205) of all cases and the relevance was mainly driven by skin contact to cosmetic products (83.2%; 124/149): Firstly rinse-off cosmetic products (38.9%), secondly leave-on cosmetic products (24.8%) and thirdly to both (19.5%). Fifteen patients (7.3%) had previously experienced allergic symptoms when being in newly painted rooms.

Conclusions

Overall, we showed that the use of MI in cosmetic products has resulted in an unprecedented epidemic of MI contact allergy. The use of MI in water-based paint is unnecessarily high and cross-reactivity between MI and OIT, and MI and BIT is likely. This is a health concern for the European citizen, justifying further preventive actions.

DANSK RESUMÉ (SUMMARY IN DANISH)

Baggrund og formål

Konserveringsmidlet methylisothiazolinon (MI) er en hyppig årsag til kontaktallergi. Det tilsættes i vid udstrækning i kosmetiske produkter og især tilsætningen i såkaldte "leave-on" kosmetiske produkter, der forbliver på huden, og vådservietter er bredt erkendt for at bidrage til et hastigt voksende antal patienter med MI kontaktallergi.

Vandbaseret maling fra det europæiske marked formodes at indeholde MI, men indtil nu har ingen eksperimentelle studier undersøgt dette. Endvidere har enkelte studier vist, at der muligvis eksisterer krydsreaktivitet mellem MI og visse isothiazolinoner, men ingen har fyldestgørende undersøgt dette.

Denne Ph.d.-afhandling har forsøgt at karakterisere og evaluere den igangværende epidemi af kontaktallergi over for MI. De enkelte formål har i detaljer været:

- At lave en retrospektiv epidemiologisk undersøgelse af kontaktallergi overfor MI og andre udvalgte konserveringsmidler hos danske eksempatienter, der blev lappetestet henover næsten tre årtier (Manuskript I).
- At analysere vandbaseret maling købt på det europæiske marked i fem europæiske lande for indhold af MI, benzisothiazolinon (BIT) og methylchloroisothiazolinon (MCI).
- At undersøge mulig krydsreaktivitet mellem MI, octylisothiazolinon (OIT) og BIT i en modificeret "local lymph node assay" (LLNA).
- At lave en prospektiv epidemiologisk undersøgelse af patienter med MI-kontaktallergi lappetestet i otte europæiske lande foruden at belyse disse patienters eksponering overfor produkter indeholdende MI.

Metode

Denne afhandling bygger på fire manuskripter. Manuskript I er baseret på en retrospektiv opgørelse af 23.138 patienter med eksem lappetestet ved Gentofte Universitets Hospital fra 1985-2013. Manuskript II bygger på kvantitativ analyse af indholdet af MI, MCI og BIT i 71 vandbaserede malinger indkøbt i fem europæiske lande. Manuskript III er baseret på kvantitativ analyse af det

immunologiske respons hos MI-sensibiliserede mus efter en eksponeringsfase med MI, OIT eller BIT. Manuskript IV bygger på prospektivt indsamlede data fra 11 centre i otte europæiske lande fra 1. maj 2015 til 30. oktober 2015.

Resultater

Introduktionen af nye konserveringsmidler på det europæiske marked har medvirket til en generel stigning i hyppigheden af kontaktallergi over for konserveringsmidler (Manuskript I). Ydermere viste vi, at relevansen af tidligere epidemiers kontaktallergi faldt signifikant over tid, mens relevansen persisterede for nuværende epidemier. Derudover fandtes MI i 93,0% (66/71) af al indkøbt vandbaseret maling i koncentrationer fra 0,7-180,9 ppm (parts per million). Der fandtes ingen forskel i koncentrationen af MI, uagtet om malingen havde miljømærkning eller ej (Manuskript II).

I vores modificerede LLNA fandt vi, at det var muligt at inducere MI-kontaktallergi. Endvidere fandt vi, at krydsreaktivitet mellem MI, OIT og BIT ikke kan udelukkes, da samme respons mhp. øretykkelse, CD4^{+} T-celler og til dels CD8^{+} T-celler blev observeret hos MI-sensibiliserede mus, der blev udsat for MI, OIT og BIT (Manuskript III). Prævalensen af MI-kontaktallergi i otte europæiske lande var 6,0% (205/3434; rangerede fra 2,6% til 13,0%) (Manuskript IV). Den hyppigste eksemlokalisation var hænder (43,4%), ansigt (32,7%), arme (14,6%) og øjenlåg (11,7%). Relevant MI-kontaktallergi blev fundet hos 72,7% (149/205), væsentligt drevet af hudkontakt til kosmetiske produkter indeholdende MI (83,2%; 124/149): Primært "rinse-off" kosmetiske produkter (38,9%), sekundært "leave-on" kosmetiske produkter (24,8%) og tertiært til begge produktkategorier (19,5%). Femten patienter (7,3%) havde tidligere oplevet luftbårne allergiske symptomer ved ophold i nyligt malede rum.

Konklusion

Summa summarum har brugen af MI i især kosmetiske produkter resulteret i en epidemi af kontaktallergi over for MI. Brugen af MI i vandbaseret maling er unødig høj og krydsreaktivitet mellem MI og OIT samt MI og BIT kan ikke udelukkes, hvilket udsætter den europæiske forbruger for en risiko.

Disse fund understreger vigtigheden af yderligere restriktioner i brugen af MI i "rinse-off" kosmetiske produkter og i forbrugerprodukter som maling.

TABLE OF CONTENTS

1. INTRODUCTION

Dermatitis is a common skin disease and could be the result of an underlying contact allergy, a so-called sensitisation to a specific allergen. Contact allergy is a T cell-mediated allergy that proceeds in two steps: (i) the phase of sensitisation where the allergen provokes an immunological T cell-mediated response in the skin and the individual becomes sensitised to this allergen; (ii) the phase of elicitation where the sensitised individual is re-exposed to the same allergen or to an allergen with chemical similarities resulting in an elicitation at the site of contact (1). The elicitation clinically manifests as allergic contact dermatitis with redness, scaling, swelling and/or vesicles. Allergic contact dermatitis is frequent in the population, and in a cross-sectional study of randomly invited individuals (18–74*yr;* n=3119) from five European countries, 27% had at least one positive patch-test result to allergens from the European Baseline Series (2).

Methylisothiazolinone (MI) is a preservative with bacteriostatic properties and its use in primarily cosmetic products has resulted in a rapid increase in new cases of contact allergy and allergic contact dermatitis (3-14). Therefore, it is important to elucidate the epidemiological and experimental aspects of contact allergy to MI.

This thesis, entitled 'Contact Allergy to Methylisothiazolinone – Observational and Experimental Studies', explores contact allergy to MI, its prevalence and the patients' exposures to products containing MI, the use of MI in water-based paint as well as cross-reactivity between MI and other common isothiazolinones. The background for the four manuscripts included in the thesis is presented in the following sections.

1.1 The immunologic mechanisms in contact allergy and allergic contact dermatitis

Contact allergy is a type IV (T cell) mediated response to an allergen. The allergenic potential of an allergen depends on (i) the allergen's capacity to penetrate the outermost layer of the skin (stratum corneum), (ii) the allergen's lipophilicity, (iii) the allergen's ability to activate the innate immune system in the skin, (iv) and the chemical reactivity (1, 15).

Contact allergy may develop after only few exposures to, for example, highly concentrated biocides in the industry or after repeated and prolonged exposures to consumer products, for

instance (16).

1.1.1 Sensitisation

The phase of sensitisation is complex and includes production of cytokines and chemokines but depends primarily on activation of allergen-specific T cells (1, 17-20).

Allergens are relatively small molecules (<500 Da; referred to as haptens) that may penetrate the stratum corneum (21). Prohaptens need activation inside the body, whereas prehaptens need activation outside the body, by UV light, for example. Upon penetration of the stratum corneum and under influence of the microenvironment's pH, the allergen conjugates with skin proteins in the deeper layers of the skin and forms sensitising compounds that may covalently bind to nucleophilic side chains such as lysine, cysteine and histidine (1, 22, 23). One crucial skin component is the major histocompatibility complex proteins (MHC class I and MHC class II molecules) abundantly present on epidermal Langerhans cells (24) that are antigen-presenting cells (APC) (1, 25).

Lipophilic haptens favour conjugation with MHC class I molecules and a later activation of CD4^{+} T cells, while hydrophilic allergens favour conjugation with MHC class II molecules and later activation of CD8^{+} T cells (26).

Resting Langerhans cells are found in the stratum spinosum. The Langerhans cells are primarily activated by activation of innate pattern recognition receptors, for example, Toll-like receptors (TLRs), either by direct allergen binding or by endogenous TLR ligands (27, 28). Upon activation, the Langerhans cells begin their migration towards the afferent lymph node and their further maturing (maturing Langerhans cells) (17, 18, 29). In the lymph node's paracortical area, the maturing Langerhans cells and naïve T cells accumulate (homing) (17, 18, 30). In the paracortical area, a cascade of T cell receptor binding between matured Langerhans cells (or other antigen-presenting cells) and naïve T cells is initiated. Activated T cells produce IL-2 and within days a thousand-fold proliferation of regulatory and effector T cells with different cytokine expression occurs in the lymph node (1, 31).

T cells can be divided into two main subsets based on their surface expression: CD4^{+} T cells ('T-helper cells') and CD8^{+} T cells ('Cytotoxic T cells') (32). CD4^{+} T cells may show different cytokine

profiles with helper/effector or regulatory/suppressive functions. Two cytokine profiles with interest for contact allergy are Th1 cells that produce IFN-γ, IL-2, and TNF-α and Th2 cells that produce IL-4, IL-5, and IL-13 (1, 33, 34). Th17 cells may further be involved (1, 33, 34). T-regulatory cells (Treg) and IL-10 secreting T-regulatory cells type 1 may also be important in the resolution of allergic contact dermatitis (35, 36). CD8^{+} T cells also show different cytokine profiles that are involved in contact allergy (1). Prolonged exposure to the allergen favours Th2-response. Regulatory B cells, for example, CD19^{+} B cells, which produce negative regulatory cytokines, such as IL-10 and TGF-β during the phase of sensitisation, are also of some interest (37).

The immune response to methylchloroisothiazolinone in combination with MI (MCI/MI) has previously been shown to elicit Th1- and Th2-type cytokines in humans with contact allergy to MCI/MI (34). It is plausible that MI alone favours the same elicitation of T cells, but this has never been investigated.

1.1.2 Elicitation

Elicitation is a delayed reaction compared with Type I hypersensitivity (immediate reaction) where mast cells degranulate within seconds to minutes. Within hours the delayed reaction is fully activated and the allergic contact dermatitis has developed. Re-exposure to the allergen activates antigen presenting cells, macrophages, mast cells and importantly keratinocytes to produce proinflammatory cytokines and chemokines that favour the migration of allergen-specific T cells (CD4^{+} and CD8^{+} T cells) from dermal vessels to the site of exposure (1, 17, 18, 38, 39). CD4^{+} T cells and CD8^{+} T cells attract neutrophils and macrophages to the site of re-exposure. Th1 cells play a crucial role in allergic contact dermatitis by producing IFN-γ that further activates inflammatory cells, for instance, macrophages (17, 18). Additionally, B-cells have been shown to activate mast cells (39). The recruitment of cells and proinflammatory cytokines and chemokines results in vascular dilation within hours and infiltration of effector cells, which initiates the allergen-specific effector phase, resulting in allergic contact dermatitis.

On the background of the aforementioned and recognized immunological actions in contact allergy, we wanted to investigate cross-reactivity between MI and other isothiazolinones based on the immune response these allergens present.

A more detailed presentation of how cytokines and chemokines influence the phases of sensitisation and elicitation is outside the scope of this thesis.

1.2 Contact allergy to preservatives

Preservatives are necessary to prevent deterioration and spoilage from microbial growth in cosmetic, household and chemical products for occupational use. Although preservatives' bacteriostatic and/or fungistatic activity is necessary, an inherent risk of developing contact allergy to preservatives exists when preservatives are in excessive contact with human skin (e.g. as part of daily grooming routines with preserved cosmetic products). Cosmetic products include a wide range of product categories: creams, deodorants, hair conditioners, hairstyling products, liquid soaps, make-up, mouthwashes, nail-care products, shampoos, shaving products, self-tanning products and so forth.

The use of preservatives has long been of concern: contact allergy to formaldehyde in the 1960s, contact allergy to methylchloroisothiazolinone in combination with MI (MCI/MI) in the 1980s, contact allergy to methyldibromo glutaronitrile in the 1990s and the early 2000s, and more recently contact allergy to MI from 2010/11 onwards (40-46). Although large retrospective studies have shown that the prevalence of contact allergy to preservatives remains relatively stable, a Danish retrospective study from 2010 found that the overall prevalence of contact allergy to selected preservatives from the European Baseline Series and Extended Series increased throughout the study period (43-45). In 1997, Dillarstone postulated that mandatory ingredient labelling of cosmetic products and post-market surveillance of contact allergy would prevent future epidemics of contact allergy (47). Notwithstanding, two epidemics of contact allergy to methyldibromo glutaronitrile and MI have since then greatly contributed to the overall burden of contact allergy to preservatives (3, 5, 6, 8-10, 12-14, 41, 42, 45). The overall prevalence of preservative contact allergy may exceed >10% in consecutive patch tested patients (45). Further, European prevalence ratios of 2–3% have been found for preservatives such as formaldehyde (with formaldehyde releasers), MCI/MI and methyldibromo glutaronitrile (43-45). Prevalence ratios of 2–3% of contact allergy may, based on CE-DUR (the Clinical Epidemiology and Drug

6

Utilization Research), for example, affect thousands of citizens in the EU (48, 49). The European Commission (EC) and health authorities set the limit for an acceptable prevalence ratio of contact allergy.

1.3 Contact allergy to methylisothiazolinone—the epidemic

Since 2010/11, the use of MI (CAS No. 2682-20-4) as a preservative primarily in cosmetic products has resulted in an unprecedented increase in the prevalence ratio of contact allergy to MI in several European countries (Fig. 1) (3-11, 13, 14, 50). Across other countries of the Western World and Asia, the rapid increase of MI contact allergy (and MCI/MI) has also been recognized; in 2013, MI was proclaimed 'contact allergen' of the year by the American Contact Dermatitis Society (51-57).

1.3.1 Introduction of methylisothiazolinone on the market

MI was introduced as a (stand-alone) preservative for use in chemical products for occupational use around 2000, when the patent of Kathon[TM] CG preservative (MCI/MI)(CAS no. 55965-84-9) expired.

In the 1980s, the use of MCI/MI in cosmetic products accounted for a rapid increase in the prevalence of contact allergy to MCI and later MCI/MI (40). However, the EC acted upon request and managed to restrict and lower the maximum permitted concentration of MCI/MI in cosmetic products, resulting in observed prevalence ratios of 1%–2% of contact allergy to MCI/MI (14, 43, 45).

In 2003, the Scientific Committee on Cosmetic Products and Non-food Products (SCCNFP; a predecessor of SCCS) came to the conclusion that the SCCNFP/0625/02 opinion that the submitted risk assessment of MI was inadequate regarding genotoxicity/mutagenicity and should be re-worked and resubmitted (58). The risk assessment of the sensitising potential of MI in the SCCNFP/0625/02 opinion was deemed adequate and no new data were therefore submitted by the industry in the second opinion on MI (59) (58, 59). At that point, new data by Basketter et al. from 2003 showed that MI (as a stand-alone preservative) had strong sensitising properties in the local lymph node assay (LLNA) (60), but this was not included in the second opinion (59) (59).

The second opinion (59) concluded that 'the proposed use of methylisothiazolinone as a preservative at a maximum concentration of 0.01% (100 ppm) in the finished cosmetic product does not pose a risk to the health of the consumer'—the use of MI at a maximum concentration of 100 ppm was later permitted (58, 59).

1.3.2 The recognition of the epidemic of contact allergy to methylisothiazolinone

Since the recognition of the rapidly increasing prevalence ratios of MI contact allergy in several European countries, national healthcare/environmental authorities, NGOs and some media have tried to alert the EC (3, 5, 6, 8-10, 12-14). Since 2010/11, the epidemic has gained additional pace with prevalence ratios of MI contact allergy of 1.5%–2.5% in 2010 increasing to 6%–12% in 2014 in European dermatitis patients (Fig. 1). In observational studies, MI contact allergy has been significantly associated with female sex, hand dermatitis, facial dermatitis and primarily work as a painter (3, 6, 8-11, 13, 14, 61, 62).

Hitherto, no European studies have estimated the prevalence ratio of MI contact allergy across European countries.

1.3.3 The restriction of methylisothiazolinone in cosmetic products

While the epidemic of MI contact allergy was gaining pace in 2014, the SCCS acted upon request in opinion SCCS/1521/13: the SCCS advised the EC to ban the use of MI in leave-on cosmetic products (including wet wipes) and to lower the use of MI in rinse-off cosmetic products to a maximum concentration of 15 ppm due to the risk of sensitisation (63). However, shortly after, the cosmetic industry requested that the EC re-evaluated opinion SCCS/1521/13. The cosmetic industry claimed that a maximum concentration of 100 ppm MI in rinse-off cosmetic products was necessary to prevent deterioration of rinse-off cosmetic products and that MI was safe for the European consumer in this concentration (64).

However, a ROAT study (ROAT: repeated open application test) showed that the use of MI in rinse-off cosmetic products in concentrations of 50 and 100 ppm MI did elicit allergic contact dermatitis in patients with MI contact allergy (65). Soon after, the SCCS concluded that the use of MI in rinse-off cosmetic products should not exceed 15 ppm (64). This final opinion (SCCS/1557/15) led to the initial legislative steps in the EC to restrict MI in leave-on cosmetic products and wet wipes (64).

Figure 1. The prevalence ratio of contact allergy to methylisothiazolinone in European countries based on consecutive patch-tested patients with suspected allergic contact dermatitis.
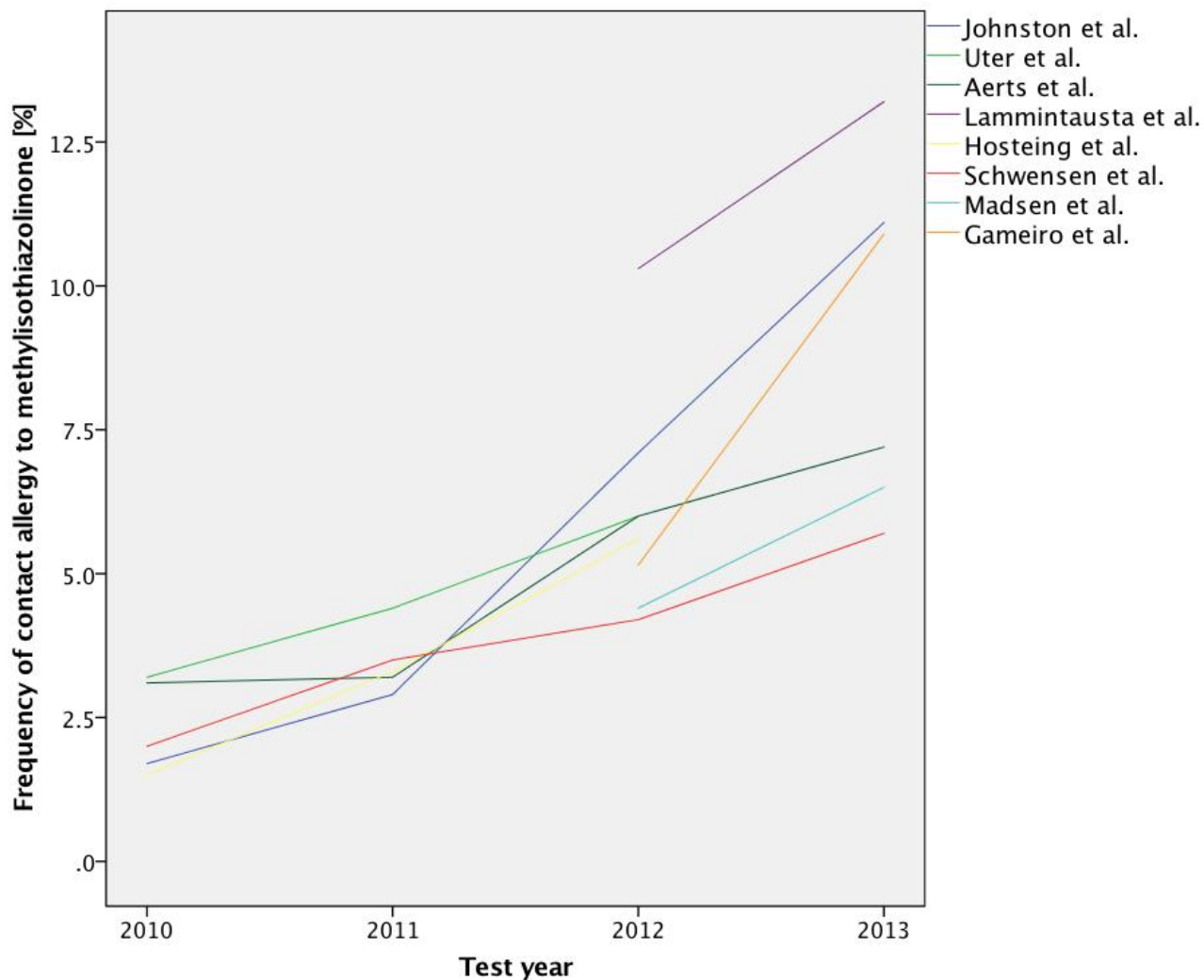
Modified from Schwensen et al. (165). Johnston et al. (9); Uter et al. (13); Aerts et al. (3); Lammintausta et al. (10); Hosteing et al. (8); Schwensen et al. (Manuscript I); Madsen et al. (12); Gameiro et al. (5).

In April 2016, the EC held the mandatory written comitology vote regarding drafting a ban on the use of MI in leave-on cosmetic products (66). The vote received unanimous agreement by all member states to support the ban. A 90-day scrutiny period followed where the European Parliament and Council were consulted before the final draft was adopted. After a 6-month transition period, MI in leave-on cosmetic products and wet wipes was banned in the EU as of 12 February 2017 (66) 2016/1198) (66).

The restriction of MI in rinse-off cosmetic products awaits action by the EC (67, 68). Therefore, it is important to explore MI exposures across European countries to recognize and gain an overview of exposures to MI.

1.4 The use of methylisothiazolinone in cosmetic products

The use of MI in cosmetic products and household products has previously been investigated. The introduction of MI in cosmetic products from 2005 and onwards led to a 25-fold increase in the maximum use dose of MI from 3.75 ppm in the previous 3:1 fixed combination in MCI/MI to 100 ppm MI as a stand-alone preservative.

In 2010, Garcia-Gavin et al. registered the first reports of MI contact allergy due to skin exposure to wet wipes and cosmetic products (46). Soon after, increasing prevalence ratios of contact allergy to MI were registered and exposure to cosmetic products containing MI was seen in 32% of patients with MI contact allergy (11). In Germany, MI contact allergy (positive patch test reactions to 500 ppm MI aq.) increased from 1.9% in 2009, 3.4% in 2010 to 4.4% in 2011 in patients patch tested with the preservative series due to suspected cosmetic contact dermatitis (6). In the following years, several observational studies showed that relevant MI contact allergy is increasing and exposure to cosmetic products (incl. wet wipes) accounts for the majority of all cases (approximately 60–70%) (3, 13, 69). Further, market surveys have shown that the occurrence of MI (and MCI/MI) in cosmetic and household products, respectively, varied between 0.5 and 7.7%, and 10.0 and 16% (69-74). In Switzerland, a recent market survey of 1266 cosmetic products showed that MI alone was found in 4.0% of cosmetic products and in 6.4% of cosmetic products intended for babies (e.g. baby wipes, creams, lotion and shower gels) (74). Notably, leave-on cosmetic products, including wet wipes containing MI, seem to pose a special risk for the consumer (3, 13, 46, 69, 73). In Italy, 94 patients with MI contact allergy, patch tested from 2012 to 2014, had ongoing allergic contact dermatitis due to skin exposures to rinse-off cosmetic products containing MI as in accordance with the aforementioned ROAT study (65, 75). Although mandatory ingredient labelling of cosmetic products exists in the EU, mislabelled MI in wet wipes has been observed (76, 77). Another case showed that MI was not labelled in a sponge for grooming (78). Accordingly, important sources of relevant MI contact allergy may be overlooked when the labelling is incorrect.

In the US, 63 disposable diaper wipes (wet wipes) and 41 topical diaper preparations were purchased in November 2015 and analysed for content of allergenic ingredients (79). It was found that MCI/MI was present in 6.3% (n=4) of all wet wipes and not observed in topical diaper preparations (79).

Despite the maximum concentration of MI in cosmetic products previously being set as 100 ppm, using high-performance liquid chromatography with ultraviolet detection, a Belgium study showed that the concentration could exceed 100 ppm in cosmetic products purchased on the Belgium cosmetic market (80). No other studies have yet verified this finding.

1.5 The use of methylisothiazolinone in chemical products for occupational use

Isothiazolinones have presumable been added to water-based paints and glues for years. In Sweden in 1998, it was found that 11.8% (9/76) of workers at a factory plant producing binders for glues and paints had contact allergy to MCI/MI (81). Later, a Swiss study showed that MCI/MI can evaporate from newly painted rooms and that MCI/MI was often used in 'paints, varnishes and coatings' as this product category covered 38.2% of 3644 identified chemical products containing MCI/MI for use in Switzerland (82). In England, contact allergy to MCI/MI and BIT has also been observed in workers at a paint manufacturer (83).

However, it was not until 2004 that Isakson et al. published two occupational cases of contact allergy to MI in two Swedish workers who had become sensitised to MI after contact with wall covering glue and after a chemical burn from a biocide, respectively (16). Soon after, a substantial outbreak of MI contact allergy in workers at a paint factory was observed in Denmark (84). In 2010, a Danish observational study showed that 1.5% of 2536 consecutive patch-tested patients had MI contact allergy, and 30% (11/30) of cases were due to exposure to chemical products at the individuals' occupational settings, for example, paint (5/11) (11). Later, more comprehensive analyses of retrospective data on patients with MI contact allergy showed that painters, machine operators, tile setters and beauticians were at special risk of developing MI contact allergy due to exposure to occupational products preserved with MI (13, 61, 62). Further, a Danish experimental study from 2014 found that the concentration of MI in 18 randomly purchased water-based paints ranged 10–300 ppm (85). However, MI is a ubiquitous preservative; accordingly, apart from its presumed use in water-based paints, it can also be found in other product categories, for example,
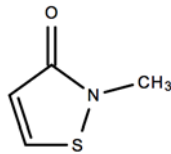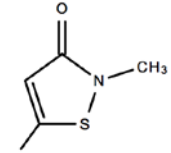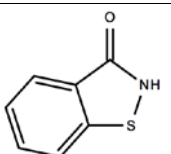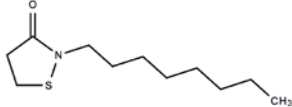
'cleaning and washing agents', 'polishing agents', 'biocides', 'softeners' and 'binding agents' (86). Additionally, evidence of undisclosed methylisothiazolinone in chemical products for occupational use has previously been published, for example, in wet wipes and in an ultrasound gel for hospital settings (50, 87, 88). Manufacturers can legally omit information on the content of MI in chemical products for occupational use because the harmonized classification of MI as a skin sensitiser in the EU awaits formal approval (89). In other non-European countries, the use of MI in chemical products for occupational use has also been found. In Japan, chemical analyses of 27 polyvinyl alcohol cooling towels showed that MI was found in the range of 0.29–154 µg g-wet(90) (91). Additionally, it has been shown that MI in water-based paints can evaporate for at least 42 days after application (85). This may result in airborne allergic contact dermatitis, probably mainly in patients already sensitised to MI (46, 92-94). Further, an observational study from Germany showed that airborne allergic contact dermatitis was associated with contact allergy to MCI/MI, which has been 'linked' to MI contact allergy since 2009 (6, 95).

The aforementioned data indicate that MI contact allergy is commonly observed after exposure to water-based paints; therefore, it is important to elucidate the use of MI in water-based paint in Europe.

1.6 Isothiazolinones

Isothiazolinones are a group of antimicrobial agents used as preservatives in cosmetic and household products and in industrial chemical products (as biocides) for more than four decades (86, 96, 97). Isothiazolinones possess bacteriostatic and/or fungistatic activity. However, isothiazolinones are also known contact allergens and the use of MCI/MI and MI has resulted in epidemics of contact allergy (7, 40, 98).

In more recent years, MI (CAS no. 2682-20-4), MCI/MI (CAS no. 55965-84-9), benzisothiazolinone (BIT; CAS no. 2634-33-5) and octylisothiazolinone (OIT; CAS no. 26530-20-1) have come to the fore as isothiazolinones of interests due to their current and/or potential use in cosmetic and household products (Table 1). Only MI and MCI/MI may be used in cosmetic products because they are included in Annex V (Table 1).

Table 1. Four selected isothiazolinones and their regulation in Annex V of Regulation (EC).

Isothiazolinone (INCI/IUPAC)	Abbreviation	CAS no.	Regulation in EU	Chemical structure
Methylisothiazolinone / 2-Methylisothiazol-3(2H)-one	MI, MIT	2682-20-4	A	
Methylchloroisothiazolinone / 5-Chloro-2-methyl-4-isothiazolin-3-one	MCI, CMI, MCIT	26172-55-4	B	
Benzisothiazolinone / 1,2-benzisothiazolin-3-one	BIT, BzI	2634-33-5	C	
Octylisothiazolinone / 2-n-octyl-4-isothiazolin-3-one	OIT, OI	26530-20-1	D	

A: Allowed in rinse-off cosmetic products up to a maximum concentration of 100 ppm. Banned in leave-on cosmetic products since 12 February 2017 (66).
B: Allowed in rinse-off cosmetic products up to a maximum concentration of 15 ppm. Banned in leave-on cosmetic products since 16 April 2016. Always in fixed combination 3:1 with MI.
C: Not included in Annex V and therefore not allowed for use in cosmetic products. In 2012, the SCCS rejected the submitted risk assessment of BIT for use in cosmetic products by the cosmetic industry (117).
D: Not included in Annex V and therefore not allowed for use in cosmetic products. The cosmetic industry has not submitted risk assessment of OIT.

Contact allergy to BIT was recognized as early as in the 1970s due to contact with a wide range of different products ranging from gum arabic to cutting oils to medical gloves (99-104). Occupational allergic contact dermatitis due to exposure to BIT in the working environment has been seen in a pottery and during the production of carpets and air fresheners (99-104). Only few retrospective observational studies exist of consecutive patients patch tested with BIT. In 1992, 1.8% of 556 Dutch dermatitis patients had contact allergy to BIT (105). In 2015, 1.6% (141/8728) of German patients who were patch tested with the metal working fluid series had BIT contact allergy (106). In Denmark, BIT contact allergy was seen in 0.4% of 3636 patients consecutively patch tested with BIT; however, with different patch test concentrations because 66.5% were

patch tested with 1000 ppm BIT aq. and 33.5% with 500 ppm BIT aq. (62). Further, BIT contact allergy has also been observed in painters and woodwork teachers (61, 62, 107-109).

In a recent Swiss market survey, 42.9% of detergents (household products) contained the following isothiazolinones: MCI, MI, BIT and OIT (74). BIT was found in 31.2% of all liquid detergents (74). Neither BIT nor OIT was found in cosmetic products (74). However, BIT contact allergy has recently been registered in a patient due to the use of a liquid soap at the workplace illegally preserved with BIT (110).

The use of OIT has been investigated in a single comprehensive study of extracted data from the Danish Product Register, a register of hazardous chemical products for occupational use in Denmark (86). Here it was found that OIT (n=111) was used less frequently than were MI (n=884), MCI (n=474), MCI/MI (n=611), and BIT (n=985) (86). OIT was primarily registered in 'paint and varnishes' (54%; 60/111) with a mean concentration of 177 ppm (86). OIT has also been found in leather products (111).

A retrospective study of 648 patients that was aimed patch tested with OIT, that is, patients under special suspicion of contact allergy to preservatives, showed that 3.1% (n=20) had a positive patch-test result to OIT (112). The majority were painters with occupational allergic contact dermatitis (112). Painters may be at particular risk of OIT (61).

1.7 Immunological cross-reactivity between isothiazolinones

Cross-reactivity between two allergens occurs when the two allergens have chemically related structures, chemical similarities. Although isothiazolinones have chemical similarities, all containing an isothiazolinone ring (Table 1), only a few and mainly observational studies have investigated cross-reactivity between MI and other isothiazolinones (3, 61, 62, 106, 113, 114). Bruze et al. has previously investigated cross-reactivity between MCI as primary sensitiser and MI in the guinea pig maximization tests (113).

The conclusion in the observational studies showed that the observed coupled reactivity may be due to co-sensitisation rather than cross-reactivity (106, 114). In 1996, Geier and Schnuch rejected that cross-reactivity between MI and BIT existed (114). However, a Belgian study from 2014 showed that the observed coupled reactions to primarily OIT in patients with MI contact allergy were not explained by a simultaneous and/or an occupational exposure to OIT but should be

ascribed cross-reactivity between MI and OIT (3). In a patient with allergic contact dermatitis on the posterior sides of both legs due to a continued exposure to MI from a newly purchased sofa, it was further found that the patient had a positive patch test reaction to OIT with no exposure to products containing OIT [Vandevenne 2014].

A small, Swedish analysis from 2008 investigating workers sensitised to MCI/MI showed that patients with a strong patch-test reaction to MCI might also react in the patch test to 1000 ppm aq. MI (115). Currently, the recommended patch-test dose of MI is 2000 ppm (116).

Further, in several observational studies the rapid increase in the prevalence ratio of MI contact allergy has subsequently increased the prevalence ratio of MCI/MI contact allergy (3, 6, 62, 69)[. It is currently unknown to what extent coupled reactivity occurs among isothiazolinone-sensitised patients, but it has previously been suggested that approximately 50% to 76% of those reacting to MCI/MI also react to MI (3, 6, 62, 69). However, observational studies are not necessarily an appropriate way to elucidate potential cross-reactivity between MI and other isothiazolinones.

It is anticipated that the cosmetic industry will be eager to replace MI with other preservatives, for example, other isothiazolinones, after MI has been/will be restricted in cosmetic products (117). Therefore, it is of utmost importance to elucidate potential cross-reactivity between MI and other common isothiazolinones.

1.8 Contact allergy to methyldibromo glutaronitrile

Methyldibromo glutaronitrile (CAS No. 35691-65-7) is a preservative with efficient antimicrobial effects and was formerly widely used in cosmetic products. In the 1980s, the EC gave permission to use methyldibromo glutaronitrile in cosmetic products (leave-on and rinse-off cosmetic products) and sunscreen products with a maximum concentration of 1000 ppm (0.1%) and 200 ppm (0.025%), respectively (118). The initial risk assessment was based on the established methods at that time (119). However, the risk assessment failed to adequately substantiate the sensitising potential of methyldibromo glutaronitrile (48, 119): (i) 11 studies with the guinea pig maximization test failed; (ii) and 7 human, repeated insult patch tests (HRIPT) also failed to demonstrate the allergenic potential of methyldibromo glutaronitrile (48, 120, 121).

In 1999, the LLNA and cumulative contact enhancement test (CCET) showed that methyldibromo glutaronitrile had sensitizing capability, especially in the permitted maximum concentration of

1000 ppm (122). Additionally, surveillance data showed in the mid- and late-1990s that the prevalence ratios of contact allergy to methyldibromo glutaronitrile had increased proportional with its use in cosmetic products and toiletries in several European countries (42, 44, 123-125). In a comprehensive observational study, the prevalence ratio of contact allergy to methyldibromo glutaronitrile in consecutive patch-tested patients with contact dermatitis (collected in 16 centres in 11 European countries) increased from 0.7% in 1991 to 3.5% in 2000 (44).

These high prevalence ratios across European countries paved the way for a re-evaluation of the sensitising risk of methyldibromo glutaronitrile. In 2002, the SCCNFP came to the conclusion that no concentration of methyldibromo glutaronitrile was safe for the European consumer in leave-on cosmetic products (126) (126). However, not until 2005 was methyldibromo glutaronitrile fully banned in leave-on cosmetic products in the EU. Later it was further recognized that rinse-off cosmetic products accounted for a substantial amount of the increase in cases with relevant contact allergy to methyldibromo glutaronitrile (127, 128). That year, in 2005, the Scientific Committee on Consumer Products (SCCP; a predecessor to the SCCS) recommended that methyldibromo glutaronitrile also should be banned in rinse-off cosmetic products as no safe concentrations could be established (129). As of 2008, methyldibromo glutaronitrile was fully banned in rinse-off cosmetic products. Decreasing trends of contact allergy to methyldibromo glutaronitrile were seen throughout the second half of 2010s (41, 43, 130).

Currently, the recommended patch-test dose of methyldibromo glutaronitrile is 0.5% (5000 ppm) pet. (116).

Surveillance data across decades can be used to describe and evaluate temporal trends of preservative contact allergy and to study the potential effects of intervention.

1.9 Risk assessment and risk management of substances in cosmetic products in the European Union

The EC governs the use of chemical substances in cosmetic products, for example, preservatives. Risk assessment refers to the pre-market procedure before a substance is granted permission for use in cosmetic products. Here, the industry submits data to support the expert opinion by the SCCS to conclude whether the substance is considered safe for use in cosmetic products in the advised concentration. After the substance is granted permission for use in cosmetic products, the

risk management is initiated. The process refers to the continuous monitoring of any adverse effects that may arise with the use of the substance in cosmetic products, for example, contact allergy. Surveillance data on contact allergy from dermatology departments and from dermatologists in private practices serve as the basis.

In the following, the legislative steps are explained more comprehensively.

1.9.1 The EU Cosmetic Products Regulation

The former "EU Cosmetic Products Directive" (76/768/EEC) and the present "EU Cosmetic Products Regulation" (Regulation (EC) No. 1223/2009) (fully applicable from July 2013) have been introduced (i) to uniform the safety of cosmetic products (cosmetics) and cosmetic substances, and (ii) to harmonize compliance within the EU Member States, simplify procedures and streamline terminology (121, 131, 132). Overall, the "EU Cosmetic Products Regulation" is a legislative framework effectuated in accordance with the overall purpose of the directive (Regulation (EC) No. 1223/2009 and former 76/768/EEC) (133). In the original "EU Cosmetic Products Directive" it was stated that no cosmetic product should cause any harm to the European consumer 'when used under normal or reasonably foreseeable conditions of use'" (Article 3) (131, 133).

1.9.2 Pre-market risk assessment and the Scientific Committee on Consumer Safety

In the European Union (EU), the European Commission (EC) is obliged to mobilize expertise to provide sufficient advice on the use of chemical substances (incl. preservatives) in cosmetic products (132, 134). This expertise is grounded in the Scientific Committee on Consumer Safety (SCCS) as it was in its predecessors: SCCP, Scientific Committee on Consumer Products; SCCNFP, Scientific Committee on Cosmetic Products and Non-Food Products; SCC, Scientific and Standardization Committee. The SCCS is an independent advisory body of DG Sante (Directorate General, Consumer Safety and Health Protection) of the EC (132, 135). The mandate of the SCCS is to provide its opinion on whether a chemical substance is safe for use in cosmetic products on the cosmetic market in the European Union (121, 132). Members of the SCCS and external experts can be toxicologists or doctors with special qualifications in the risk assessment process of chemical

substances that justify their presence as members of the SCCS (135). An opinion of the SCCS is based on evaluation of the chemical substance's toxicological dossier submitted by the industry (132).

The European Parliament and member states may thereafter approve chemical substances (with a positive opinion) for use in cosmetic products on the European cosmetic market. All chemical substances for use on the European market in cosmetic products are listed in Annex V of the EU Cosmetic Products Regulation (Regulation (EC) No. 1223/2009) (121, 133). Only preservatives listed in Annex V are allowed for use in cosmetic products in the European Union (121, 133).

1.9.3 Post-market risk assessment and surveillance data

While the aforementioned pre-market risk assessment of substances is based on an at-that-time-acceptable approach to risk evaluate a new substance for use in cosmetic products, the post-market risk management of substances is based not only on novel research, but also primarily on clinician-driven surveillance data of contact allergy (136-138). The EU Cosmetic Products Regulation (Regulation (EC) No. 1223/2009) states that the safety of a cosmetic product on the cosmetic market is with the designated "responsible person" (legal person) (Articles 4 and 5), and member states have a legal obligation to entrust market surveillance authorities with the necessary powers to monitor this compliance (Article 22) (121, 133). In matters of substances in cosmetic products causing harm to the European consumer (a breach of Article 3), competent (national) authorities shall immediately (i) take provisional measures, (ii) communicate this concern to the EC, (iii) and further communicate this concern and the measures taken at a national level to the competent authorities of the other member states (Article 27) (121, 133).

2. METHODS: THE NATIONAL ALLERGY RESEARCH CENTRE AND STUDY POPULATIONS

2.1 The National Allergy Research Centre

The Ministry of Environment founded the National Allergy Research Centre in 2001. In relation to the founding of the National Allergy Research Centre and with the aid of the well-established

network of dermatologists in the university hospitals' dermatology departments and dermatologists in private practice, the surveillance database of contact allergy 'National Database of Contact Allergy' was formed. Its purpose is to continuously monitor frequencies of contact allergy and clinical data for patients with dermatitis treated at university hospitals' dermatology departments and dermatologists in private practice. Accordingly, the National Allergy Research Centre is engaged in research and the continuous surveillance of the prevalence ratio of contact allergies in the population.

Data from a single centre may also be extracted for research purposes as the systematic registration of data for contact allergy dates back to 1985 for the Department of Dermatology and Allergy, Copenhagen University Hospital, Herlev-Gentofte, while for others it is an activity more recently initiated.

2.2 The modified local lymph node assay

The local lymph node assay (LLNA) was originally made for hazard identification and as a measurement of relative potency (139). Groups of CBA mice are by topical application of the dorsum of both ears exposed to the allergen in various concentrations or to a vehicle control for three consecutive days (139). On Day 5, mice are given an intravenous injection of tritiated thymidine (3H-TdR) and killed 5 hours later. The draining lymph nodes are then excised (139). Often, a positive control of hexyl cinnamic aldehyde is included in the setup. Data are pooled of each experimental group or experimental animal basis and processed for β-scintillation, counting of the cells in the draining lymph nodes. A stimulation index (SI) is thereafter calculated for each substance. A skin sensitiser is defined here as the concentration of a substance that induces a threefold increase in the cells of the draining lymph nodes (SI of 3; EC3) (139).

The modified local lymph node assay (Fig. 2) is described in detail in Manuscript III.

Figure 2. (a) The sensitisation phase in the modified local lymph node assay and (b) the challenge phase in the modified local lymph node assay.
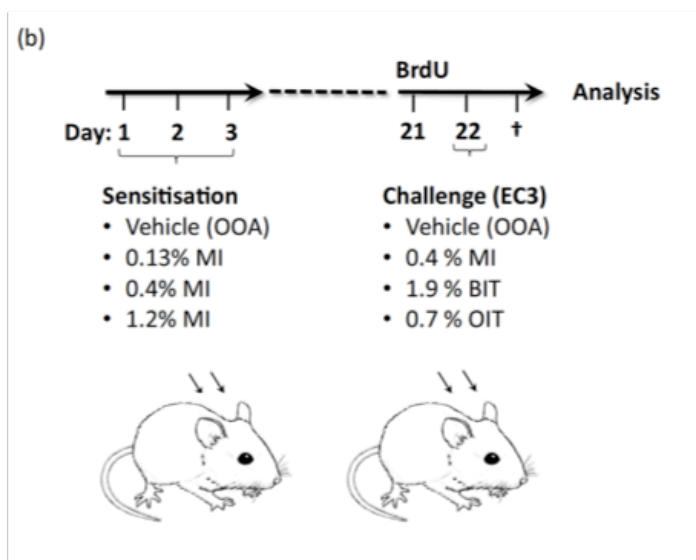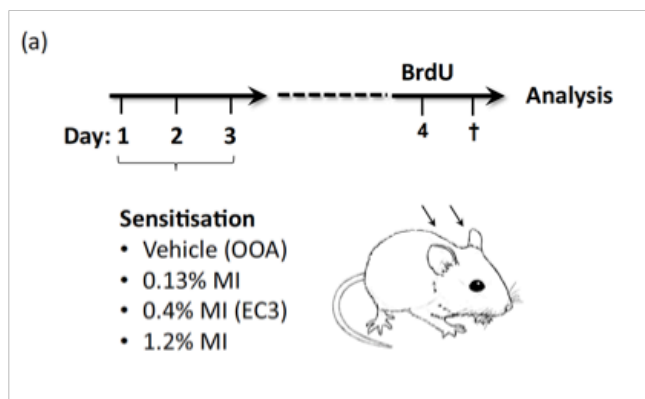
Ear thickness was measured by engineer micrometre on Day 5 in sensitisation phase (a) and on Day 23 in challenge phase (b). The draining local lymph node(s) were then removed for flow cytometry for measurement of CD4$^+$ T cells, CD8$^+$ T cells and CD19$^+$ B cells.

2.3 Ethics statement

Manuscript I is based on anonymized data from the 'National Database of Contact Allergy'. In

Manuscript III, all mice were housed in accordance with national animal protection guidelines (licence number 2012-15-2934-00663). No ethics statement was relevant for Manuscript III.

In Manuscript IV, all participants signed a written informed consent form before inclusion in Study IV. Further, the storage of Danish data for Study IV was approved by the Danish Data Protection Agency (GEH-2015-076, I-suite no. 03709). All other Centres followed their regional/national guidelines for storage of data and only anonymous data were sent to the National Allergy Research Centre for inclusion in the study.

2.4 Statistical analyses

Statistical analyses were performed using (i) SPSS[TM] Statistics, Chicago, IL, USA, IBM PASW Statistics for Windows[TM] and Mac OS X, edition 19.0 and 20.0, (ii) R statistical software (version 3.1.0; www.r-project.org) and (iii) RStudio (Version 0.98.1103 for Mac OS X). Statistical analyses for dichotomous variables were done using the Chi Square test and Fishers Exact test when appropriate. Continuous variables were presented as mean when data were normally distributed and as median scores with interquartile range (25th and 75th percentiles) when data were non-normally distributed. Normal distribution was assumed only after visual inspection of histogram, and Kolmogorov-Smirnov test and/or Shapiro-Wilk test for normal distribution. In Manuscript III normal distribution was assumed after log transformation.

The distribution was graphically represented with either strip charts with means or with strip charts with overlay boxplots.

The statistical threshold for statistical significance in all studies was predefined as p-value < 0.05.

In Manuscript I, the Chi Square test linear-by-linear association was utilized to test for trends of preservative contact allergy across test years. Binary logistic regression analysis was used to ascertain the effects of background variables (MOALHFA-index) on the development of preservative contact allergy (dependent variable). Associations were expressed as odds ratios (OR) with 95% confidence intervals (CI).

In Manuscript II, non-parametric variables were tested for group differences using Kruskal-wallis-H test for global heterogeneity. Additional post-hoc pair-wise testing with Mann-Whitney U-test was applied between selected groups.

In Manuscript III, preselected one-way ANOVA with post hoc Tukey's honest significant difference

(HSD) test for global heterogeneity was applied for analysis of differences in means across subgroups.

Figures were made in SPSS, R statistical software, molecular structures in ChemSpider (http://ChemSpider.com), and maps in P&P World Map (http://edit.freemap.jp/en/trial_version/edit/europe). Figures were later modified in Adobe Photoshop CC®.

3. OBJECTIVES OF THE STUDIES

Study I

- To describe and evaluate temporal trends of preservative contact allergy.
- To characterize and evaluate previous and present epidemics of preservative contact allergy and effect of intervention.

Study II

- To determine the concentrations of MI, MCI and BIT in water-based wall paints purchased in retail outlets for analysis of consumer exposure.
- To explore environmental labelling of water-based paints regarding MI.

Study III

- To induce contact allergy to MI in mice.
- To investigate whether MI sensitised mice develop the same immune response regarding CD4^{+} T cells, CD8^{+} T cells and CD19^{+} B cells when challenged with MI as with OIT and BIT.

Study IV

- To characterize European patients with MI contact allergy during a defined period of 6 months.
- To identify their exposures to cosmetic products, household products, and industrial chemical products containing MI.

4. RESULTS AND MANUSCRIPTS

This section summarizes key findings related to the stated objectives. The original manuscripts are included after each summary.

Manuscript I is based on data from Herlev-Gentofte University Hospital in Denmark. Manuscript II is based on chemical analysis of purchased water-based paint from five European countries. Manuscript III is based on data from a modified local lymph node assay in mice. Manuscript IV is based on data from 11 European centres that prospectively collected data for six months on patients with MI contact allergy.

4.1 Failures in risk assessment and risk management for cosmetic preservatives in Europe and the impact on public health – Manuscript I

- The prevalence of preservative contact allergy in a uniform retrospective cohort of patients patch tested in a university hospital significantly increased from 6.7% in 1985 to 11.8% in 2013.

- Methyldibromo glutaronitrile remained relatively high (3–6%) after 1999 where methyldibromo glutaronitrile was introduced as part of the baseline series.

- The present clinical relevance of methyldibromo glutaronitrile decreased from >90% in 1999 to <10% in 2013.

- The prevalence of MI significantly increased from 1.5% in 2005 to 5.7% in 2013 (p<0.001).

- The clinical and present relevance of MI contact allergy remained stable at approximately 60–80% during 2005–2013.

- Facial dermatitis affected approximately 20–25% of all patients with preservative contact allergy between 2001 and 2009 and showed a steep increase thereafter up to approximately 40%.

- This increase was mainly due to MI contact allergy and the adjusted attributable risk percentages associated between facial dermatitis and MI contact allergy were 40% and 49% during 2010–2013 and the test year 2013, respectively.

Failures in risk assessment and risk management for cosmetic preservatives in Europe and the impact on public health

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Summary

Background. In view of the current and unprecedented increase in contact allergy to methylisothiazolinone (MI), we characterized and evaluated two recent epidemics of contact allergy to preservatives used in cosmetic products to address failures in risk assessment and risk management.

Objective. To evaluate temporal trends of preservative contact allergy.

Methods. The study population included consecutive patch tested eczema patients seen at a university hospital between 1985 and 2013. A total of 23 138 patients were investigated for a contact allergy.

Results. The overall prevalence of contact allergy to at least one preservative increased significantly over the study period, from 6.7% in 1985 to 11.8% in 2013 ($p < 0.001$). Importantly, the preservatives methyldibromo glutaronitrile and MI rapidly resulted in high sensitization prevalence rates, which reached epidemic proportions. Although the proportion of patients with current clinical disease attributable to methyldibromo glutaronitrile contact allergy decreased significantly following the ban on its use in cosmetic products ($p < 0.001$), the sudden and high proportion of current sensitization to MI requires immediate attention ($p < 0.001$).

Conclusions. The introduction of new preservatives in Europe with inadequate pre-market risk assessment has rapidly increased the overall burden of cutaneous disease caused by preservatives. We suggest that the cosmetic industry has a responsibility to react faster and replace troublesome preservatives when a preservative contact allergy epidemic is recognized, but the European Commission has the ultimate responsibility for failures in risk management after new, major sensitizing preservatives are introduced onto the market.

Key words: allergic contact dermatitis; epidemic; methyldibromo glutaronitrile; methylisothiazolinone; preservatives; risk management.

Preservatives are used in cosmetic, household and industrial chemical products (when they are referred to as 'biocides') to prevent microbial growth and spoilage. Although only a minor proportion of the general population is in daily and repetitive skin contact with preserved industrial chemical products, daily exposure to preservatives in cosmetic products (personal care products and toiletries), such as moisturizing lotions, shampoos or skin cleansers, and household products, is a common part of daily routines. Excessive exposure to allergenic preservatives may cause contact allergy and allergic contact dermatitis, a skin condition that can become chronic and only resolves if contact with the allergen is avoided.

In a historical perspective, the European Commission (EC) has accepted the need to deviate from the intention of the Cosmetics Regulation (previously Directive) by permitting the use of preservatives with significant sensitizing capacity in cosmetic products, as there is a reasonable demand and need for product preservation. The mandate of the Scientific Committee on Consumer Safety (SCCS) and its predecessors, an independent advisory body of DG Sante (Directorate General, Consumer Safety and Health Protection, previously known as DG Sanco) of the EC, is to provide its opinion on the question of whether the use of a chemical substance, for example a preservative, is safe for the consumer in cosmetic products from a public health point of view (1). Within the EU, only those preservatives on a 'positive list' annexed to the Regulation may be used in cosmetic products. Opinions from the SCCS on questions concerning the sensitizing capability of a preservative are based on predictive experimental assays, animal studies (which are no longer permitted if the experiments are to be performed solely to provide data required for safety evaluation of a substance for cosmetic use), and, partly, human testing with standardized methods (2–4). Opinions at the time of assessment (when the opinions are formed) are not based on large amounts of clinical and epidemiological data, as these data are generated after marketing of a preservative. Subsequent reassessment of a preservative (risk management) can be triggered by these post-marketing surveillance data when issues arise.

In 1989, de Groot and Herxheimer recognized the growing problem of contact allergy to the widely used preservative methylchloroisothiazolinone (MCI) in the fixed 3:1 combination with methylisothiazolinone (MI) (MCI/MI) (5). It was predicted that future preservative contact allergy epidemics could potentially be avoided by (i) adequate risk assessment, (ii) mandatory ingredient labelling of cosmetic products, and (iii) prioritized detection of new sensitizing preservatives (5, 6). It was foreseen that these reasonable initiatives would result in safer cosmetic products and prevent the emergence of new preservative contact allergy epidemics.

In view of the unprecedented epidemic of contact allergy to the recently marketed preservatives methyldibromo glutaronitrile and MI, and what seems to be a trend of recurring epidemics of contact allergy to preservatives, we find it important to (i) describe and evaluate temporal trends of preservative contact allergy, and (ii) characterize and evaluate previous and present epidemics of preservative contact allergy in order to propose a better risk management procedure for novel and current preservatives in EU member states.

Materials and Methods

The study population included consecutive patients with dermatitis (eczema) who underwent routine diagnostic patch testing at Copenhagen University Hospital Gentofte between 1 January 1985 and 31 December 2013 for contact allergy. All patients were patch tested with at least the European baseline series of contact allergens and additional allergens from extended test series (7).

Contact allergy information on the following and most frequent preservatives was extracted from the database: formaldehyde (CAS no. 50-00-0), formaldehyde releasers [2-bromo-2-nitropropane-1,3-diol (CAS no. 52-51-7), diazolidinyl urea (CAS no. 78491-02-8), DMDM hydantoin (CAS no. 6440-58-0), imidazolidinyl urea (CAS no. 39236-46-9), and quaternium-15 (CAS no. 4080-31-3)], iodopropynyl butylcarbamate (CAS no. 55406-53-6), methyldibromo glutaronitrile (MG) (CAS no. 35691-65-7), MCI/MI (CAS no. 55965-84-9), MI (CAS no. 2682-20-4), and paraben mix [methylparaben (CAS no. 99-76-3), ethylparaben (CAS no. 120-47-8), propylparaben (CAS no. 94-13-3), and butylparaben (CAS no. 94-26-8)]. All patients were patch tested with all of the preservatives after their inclusion in the diagnostic patch test series.

Available information from the database included age, the baseline characteristics of patients according to the MOALHFA index (information on male gender, occupational relevance of a contact allergy, atopic dermatitis, leg dermatitis, hand dermatitis, facial dermatitis, and age > 40 years), and the outcome of patch testing. However, the MOALHFA index in its present form was not routinely registered throughout the entire study period. Thus, information on atopic dermatitis and information on facial dermatitis were not collected until 1994 and 2001, respectively.

Patch tests with allergens were applied to the upper back. The occlusion time was 2 days, and readings were performed after 2, 3 (or 4) and 7 days, in accordance with

Table 1. The distribution of the MOAHLFA index for 21 247 eczema patients with no contact allergy to preservatives and 1891 patients with contact allergy to at least one preservative

	Cases with no contact allergy to preservatives, % (n)	Cases with contact allergy to at least one preservative, % (n)	OR (95%CI)	*p*-value
Male	34.9 (7418)	31.6 (598)	0.82 (0.74−0.91)	< 0.001
Occupational contact dermatitis	11.1 (2354)	16.5 (312)	1.09 (0.94−1.27)	0.245
Atopic dermatitis	11.0 (2340)	10.3 (194)	0.83 (0.71−0.98)	0.030
Hand dermatitis	23.9 (5076)	40.1 (758)	2.31 (2.07−2.59)	< 0.001
Leg dermatitis	5.0 (1061)	6.7 (126)	1.28 (1.05−1.56)	0.013
Facial dermatitis	10.6 (2251)	15.1 (286)	1.57 (1.37−1.80)	< 0.001
Age > 40 years	63.7 (13 544)	76.5 (1447)	2.00 (1.78−2.24)	< 0.001

OR (95% CI), odds ratio with 95% confidence interval obtained from binary logistic regression modelling.

ICDRG recommendations (8). Reactions of strength 1+, 2+ and 3+ were interpreted as positive responses. Irritant reactions, doubtful reactions and negative reactions were interpreted as negative responses. In cases of repeated testing, patch test data from the last visit were used in the analysis.

'Relevance' was defined as a current and certain association between contact allergy and the clinical manifestation of contact dermatitis.

An 'epidemic' is generally a definition used for infectious diseases. The term 'contact allergy epidemic' has, in dermatological conditions such as contact allergy and allergic contact dermatitis, been defined as the occurrence of disease in a geographical region or within a specific population that is in excess of that normally expected (9).

Statistics

All data analyses were performed with SPSS™ version 19.0 (SPPS™ Statistics Chicago, IL, USA; IBM PASW Statistics) for Windows™. Binary logistic regression analysis was performed to ascertain the effects of background variables (MOALHFA index), that is, explanatory variables, on the likelihood of developing preservative contact allergy (dependent variable). The χ^2-test linear-by-linear association was utilized to test for trends of preservative allergy across test years. Attributable risk percentage was used to test the contribution of MI contact allergy to the development of facial dermatitis. All *p*-values are two-sided, and 0.05 was chosen for statistical significance.

Results

A total of 23 138 patients aged 1−100 years were patch tested between 1985 and 2013. The MOAHLFA index for patients without preservative contact allergy (n = 21 247) and patients with contact allergy to at least one preservative (n = 1891) is shown in Table 1. Atopic dermatitis was only registered between 1994

and 2013, and facial dermatitis between 2001 and 2013. Hand dermatitis, facial dermatitis and older age (age > 40 years) were all highly significantly associated with having contact allergy to at least one preservative (*p* < 0.001).

Facial dermatitis affected approximately 20−25% of tested patients with preservative contact allergy during 2001−2009, but showed a steep increase to 41% of patients in 2013 (*p* < 0.001). When patients with MI contact allergy were excluded, the increase in facial dermatitis after 2009 appeared to be less dramatic, affecting only 28% (*p* = 0.025) in 2013. The MI contact allergy adjusted attributable risk percentages associated with facial dermatitis were 40% and 49% for the period 2010−2013 and the test year 2013, respectively. This observation suggests that MI contact allergy had a strong impact on the increasing prevalence of facial dermatitis seen in recent years.

Figure 1 shows the temporal trends of contact allergy to preservatives. Notably, the overall prevalence of sensitization to at least one preservative increased from 6.7% in 1985 to 11.8% in 2013 (*p* < 0.001), suggesting an increased burden of preservative contact allergy. Whereas methyldibromo glutaronitrile contact allergy showed a relatively high prevalence, ranging between approximately 3% and 6%, the clinical relevance of methyldibromo glutaronitrile contact allergy, that is, the proportion with allergic contact dermatitis caused by methyldibromo glutaronitrile, decreased significantly, from >90% of cases between 1999 and 2004 to <10% of cases in 2013, following the ban on its use cosmetic products in Europe (Fig. 2; *p* < 0.001).

Importantly, the prevalence of MI contact allergy increased from 1.5% in 2005 to 5.7% in 2013 (*p* < 0.001), making MI the single most problematic preservative after 2010 (Fig. 1). The clinical relevance of positive patch test reactions to MI became high immediately after its inclusion in the patch test series, and remained stable across the test period, with no significant
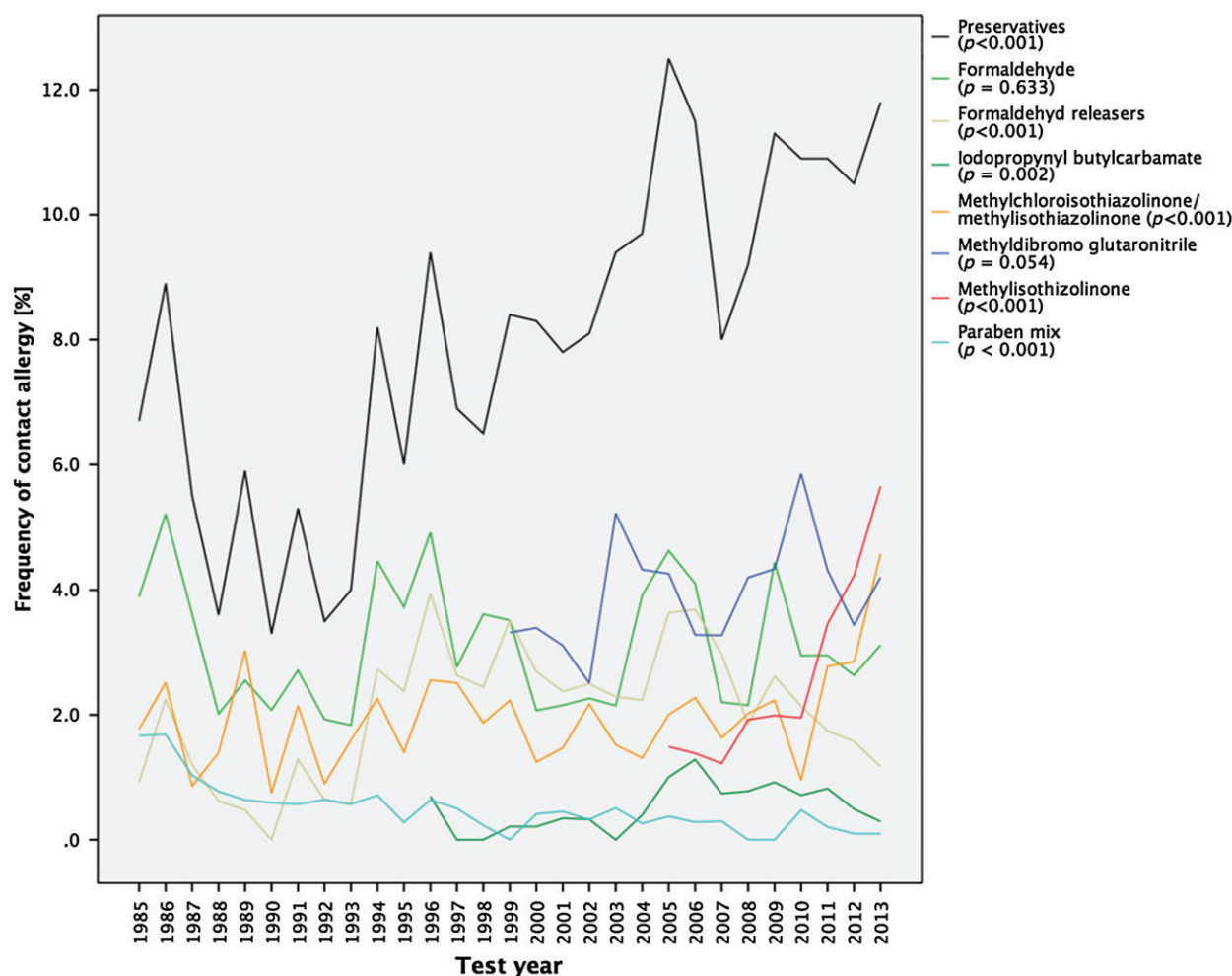
Fig. 1. Temporal trend of preservative contact allergy of 23 138 patients suspected of having allergic contact dermatitis eveluated at a hospital university clinic in Copenhagen between 1985 and 2013. Formaldehyde releasers: 2-bromo-2-nitropropane-1,3-diol, diazolidinyl urea, DMDM hydantoin, imidazolidinyl urea, and quaternium-15. 'Preservatives': Contact allergy to at least one preservative.

trend, and 60–80% of positive reactions being considered of current clinical relevance by the dermatologist (Fig. 3).

Discussion

This 29-year retrospective epidemiological study investigated the recurring epidemics of preservative contact allergy among consecutively patch tested patients with dermatitis. Our data suggest that, each time a new preservative has been marketed, it has added to the overall prevalence, and probable burden, of contact allergy to preservatives; for example, the epidemics of methyldibromo glutaronitrile and MI contact allergy contributed with a prevalence of 4–6% within the period 1999–2013 (10). These recurring epidemics of preservative contact allergy represent a public health challenge,

as thousands of dermatitis patients across Europe have developed lifelong allergies to these intensively used preservatives following exposure to cosmetic products in particular (5, 10–17). Affected individuals may develop occupational skin problems because of secondary exposure in the work environment, resulting in sick-leave and, ultimately, retraining. Children may also develop contact allergy to preservatives, with an impact on their well-being. For example, MI contact allergy after exposure to wet-wipes or to sunscreen preserved with MI has resulted in nappy and facial dermatitis, respectively (18, 19).

Collectively, these data emphasize that it is crucial, for personal and economic reasons, to carefully consider and evaluate the risk assessment process prior to introducing new preservatives. The two most recent epidemics of methyldibromo glutaronitrile and MI contact allergy

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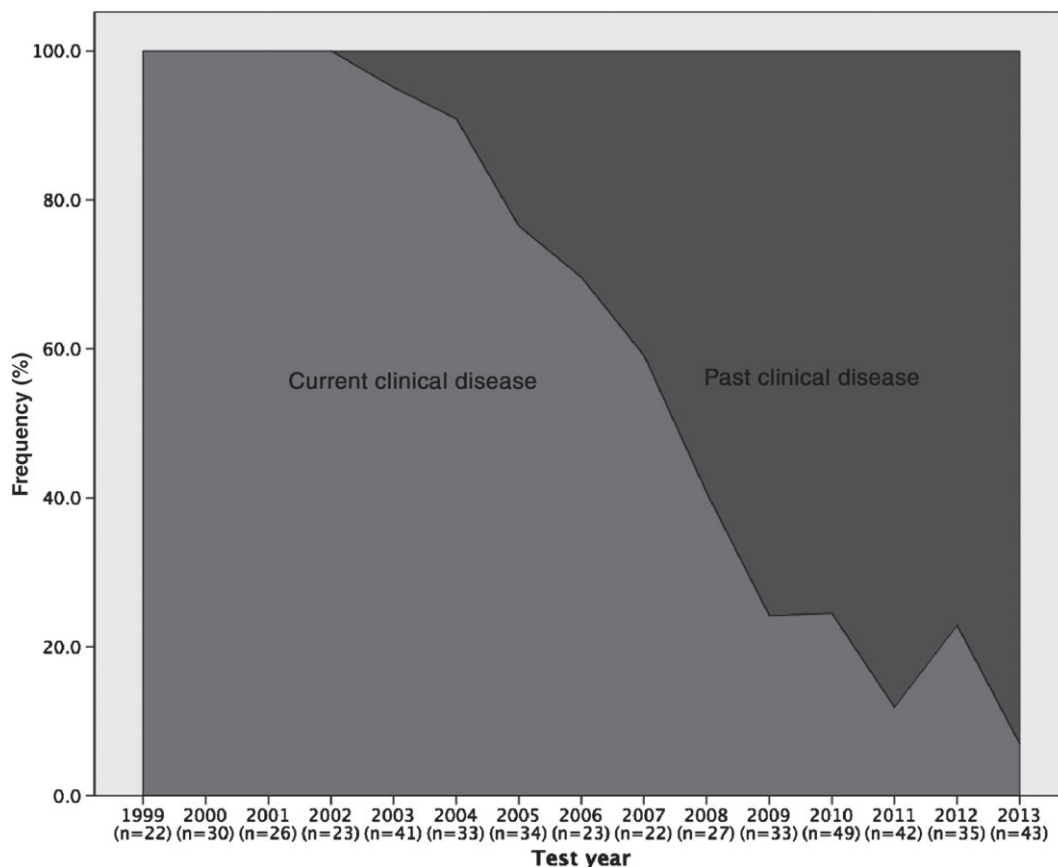
Fig. 2. Time trend based on the frequency of clinical relevance of methyldibromo glutaronitrile contact allergy for 483 patients with methyldibromo glutaronitrile contact allergy between 1999 and 2013.

illustrate the weakness in the process of permitting these major sensitizing preservatives.

Risk assessment

The former 'EU Cosmetic Directive' (76/768/EEC) and the 'EU Cosmetic Products Regulation' (Regulation No. 1223/2009) applied from 2013 state that no cosmetic products should cause any damage to human health when applied under normal or reasonable foreseeable conditions of use (1, 20).

Ultimately, the cosmetics industry is responsible for substantiating the safety of cosmetic products and chemical substances, including preservatives, used in their products. However, preservatives used in cosmetic products in Europe are on a 'positive list' in the legislation, and industry may only use a preservative if it is permitted in the list. Permitted preservatives must first be assessed for safety by the SCCS; industry is responsible for providing the dossier of data required for formal assessment by the SCCS. Failure to provide adequate data to enable formal

risk assessment will lead to a negative opinion of the preservative, and the preservative will not be permitted for use in cosmetic products. The deficiency in risk assessment concerning contact allergy to preservatives before they are introduced in cosmetic products is evident: several epidemics of preservative contact allergy have emerged, namely formaldehyde in the 1960s, MCI/MI in the 1980s, methyldibromo glutaronitrile in the late 1990s, and now the recent and unprecedented epidemic of MI contact allergy (3, 10, 11). Pre-market risk assessment of the safety of chemical substances is based on accepted toxicological approaches. However, post-market risk management and re-evaluation require surveillance to detect problems once the consumer is being exposed to a preservative, and then action to reduce exposures should contact allergy to the preservative become an issue post-marketing. The problems with the current risk assessment methodology and the dramatic failure of risk management are of public health concern. The procrastination of the EC as a risk manager has been discussed previously (21, 22).
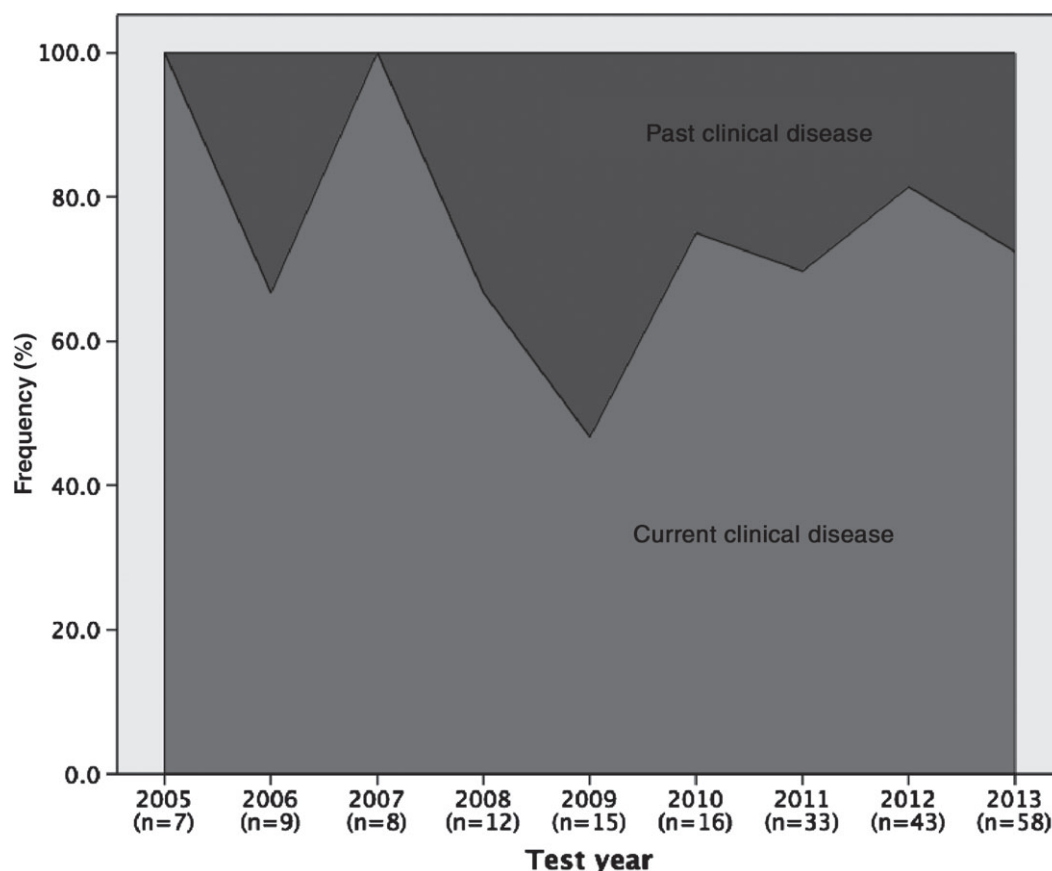
Fig. 3. Time trend based on the frequency of clinical relevance of methylisothiazolinone contact allergy for 201 patients with methylisothiazolinone contact allergy between 2005 and 2013.

Risk assessment and risk management for methyldibromo glutaronitrile

The rise in methyldibromo glutaronitrile contact allergy prevalence throughout Europe in the late 1990s should have alerted the EC at an earlier point regarding better risk management of new preservatives (23, 24).

The legal adoption of methyldibromo glutaronitrile in cosmetic products in a concentration up to 0.1% was based on established methods for evaluating the allergenic potential of a chemical (such as the guinea-pig maximization test), but these failed to show its allergenic potential (25, 26). Later animal studies with multiple topical applications during the sensitization phase showed methyldibromo glutaronitrile to be a sensitizer. Clinical and epidemiological studies on patients with dermatitis showed an increasing frequency of contact allergy to methyldibromo glutaronitrile and resulting allergic contact dermatitis (23, 24, 27). In 2002, the SCCS recommended that methyldibromo glutaronitrile should no longer be used in leave-on cosmetic products

(SCCNFP/0585/02) and, in 2005, the SCCS recommended that it should no longer be used in rinse-off cosmetic products (SCCP/0863/05), as no safe concentrations for methyldibromo glutaronitrile could be established (28, 29). This led to a significant reduction in the clinical relevance of methyldibromo glutaronitrile, but the prevalence of contact allergy to methyldibromo glutaronitrile remains high.

Risk assessment and risk management for MI

In the early 2000s, MI was introduced as a stand-alone preservative for use in industrial chemical products, with no upper limit in concentration, for example as a preservative for use in glue or paint (19, 30).

The SCCS stated in 2003, in their first opinion on MI (SCCNFP/0625/02), that no adequate risk assessment of MI could be carried out, as the genotoxicity/mutagenicity studies were inadequate (31). The issue of sensitization to MI was considered to be adequately addressed at the time of the first opinion, and no

29

Table 2. Overview of adequate risk assessment of pre-marketed preservatives and future approaches to avoid epidemics of contact allergy to preservatives used in cosmetic products

Pre-market risk assessment of preservatives	Marketed preservatives
Industry:	
Transparent and reproducible *in vivo*, *in vitro* and *in silico* testing	Obligation to use less sensitizing allergens, e.g. acids
	The use of preservatives in combination to lower the concentrations rather than the use of a single preservative in a high concentration
Legal authorities:	
Well-carried-out risk assessment	Authorities grant temporary permission for the use of newly marketed preservatives in cosmetic products. Re-evaluation and granting of permanent permission take place in a short time frame, e.g. 5 years, to avoid the permanent use of highly sensitizing preservatives
- Risk-based approach to the processes of risk assessment and risk management: a distinction between high (leave-on) and low (rinse-off) exposures	–
- Any occupational use of the preservative and occupationally related allergic contact dermatitis to the preservative must be considered, as it may provide information about a highly sensitizing and troublesome preservative	Derogation of highly sensitizing preservatives
- Strong or extreme sensitizers should not be permitted	–

new data regarding sensitization to MI were submitted by industry for inclusion in the second opinion on MI (SCCNFP/0805/04) (31, 32). Using the local lymph node assay, Basketter et al. showed in 2003 that MI has strong sensitizing capabilities, but this information was not included in the second opinion, as the paper was not provided in the dossier submitted by industry, and no third process in the risk assessment of MI was triggered by these data (32, 33). Of concern is the conclusion drawn from the human repeated insult patch tests conducted by the industry that the threshold level for sensitization is well above what was to be the permitted concentration of 100 ppm MI in cosmetic products (32). The opinion on MI (SCCNFP/0805/04) stated that 'the proposed use of methylisothiazolinone as a preservative at a maximum concentration of 0.01% (100 ppm) in the finished cosmetic products does not pose a risk to the health of the consumer' (32).

Since 2010, the prevalence of MI contact allergy has increased at an alarming rate, and several European countries have confirmed the existence of an MI epidemic, primarily because of its use in cosmetic products, with prevalence rates of more than 6–12% in consecutively tested contact dermatitis patients in many European countries (12–17). In light of the prevailing tendency for there to be MI contact allergy among patients with contact dermatitis, in 2011 European dermatologists raised their concern on the use of MI in cosmetics (34).

Until 2013, MI was stated and erroneously recognized as possessing moderate sensitizing capabilities, based on a review article of compilations of potency values, that is,

reporting modelling studies on a large set of local lymph node assay data (35, 36). However, MI actually possesses strong sensitizing capabilities, as pointed out in an editorial by Roberts (33–37).

The current epidemic of MI contact allergy in several member states of the EU prepared the ground for a revision of the opinion on MI (SCCS/1521/13), published in December 2013. The SCCS concluded that no safe level of MI had been determined for leave-on cosmetic products (e.g. lotions and wet-wipes), and that, for rinse-off cosmetic products (e.g. soaps and shampoos), a maximum concentration of 15 ppm MI was safe from the point of view of sensitization (34). Hitherto, the EC has not acted upon the advice of its independent advisory committee, but has acted on the request of industry to ask the SCCS to re-evaluate its opinion that MI is not safe for use in rinse-off products and hair care products at 100 ppm, as described above (22).

Epidemics and the impact of regulatory interventions

The introduction of regulatory interventions for these highly problematic preservatives will not lead to a decreasing prevalence of morbidity for some years, as implementation of prohibitions/restrictions is delayed, the market is allowed to sell off already manufactured cosmetic products, cosmetic products purchased before the prohibition/restriction may still be in the possession of consumers, and the population of already sensitized (and not recognized) individuals is alarmingly large. Fully implemented regulatory interventions will nevertheless lead to decreasing clinical relevance of the restricted

preservative. Our data indicate that the background population with methyldibromo glutaronitrile contact allergy may be large and not yet fully recognized, as the prevalence of contact allergy to methyldibromo glutaronitrile at this stage remains unchanged, whereas the clinical relevance of methyldibromo glutaronitrile has diminished (Figs. 1 and 2).

It is important to emphasize that patients remain with lifelong contact allergy to the preservative, and will, with appropriate exposure to the preservative in domestic or occupational settings, develop allergic contact dermatitis. Furthermore, the prohibition of these preservatives is seldom internationally applied, and the consumer may be therefore exposed to preservatives whose use is prohibited in the EU, but that are allowed for preservation in cosmetic products in other parts of the world.

It is suggested that the EC, as the legal authority and risk manager, should grant only temporary permission for use of a newly approved preservative for use in cosmetic products. Re-evaluation and/or more permanent permission for use of the preservative would be granted after the preservative had been present on the market, and the frequency of any developing contact allergy to it in the consumer had been evaluated.

In conclusion, the EC should be a more active manager when industry presents a safety dossier of a preservative for risk assessment; strong or extreme sensitizers should, as a point of departure, not be acceptable for use in cosmetic products. There are currently available a number of preservatives permitted for use in cosmetic products that should give manufacturers ways to adequately preserve cosmetics without using extreme or strong sensitizers, for example the less allergenic acids.

We therefore suggest that (i) meticulous and adequate risk assessment of contact allergens should be prioritized in the future, (ii) temporary permissions and later re-evaluation should be introduced by legal authorities to avoid delay in the derogation of recognized and highly allergenic preservatives, and (iii) the cosmetic industry should accept responsibility for reacting faster and replacing troublesome preservatives when a developing epidemic of contact allergy to a preservative epidemic is recognized (Table 2). The recurring epidemics of contact allergy to preservatives represent a concern for public health.

Reference

1 European Commission. Council Directive of 27 July 1976 on the approximation of the laws of the Member States relating to cosmetic products (1976L0768-EN-24.04.2008-021.002-3). Available at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=CONSLEG:1976L0768:20080424:en:PDF (last accessed 09 November 2014).

2 Thyssen J P, Giménez-Arnau E, Lepoittevin J-P, Menné T, Boman A, Schnuch A. The critical review of methodologies and approaches to assess the inherent skin sensitization potential (skin allergies) of chemicals Part I. *Contact Dermatitis* 2012: **66**: 11–24.

3 Menné T, Wahlberg J E, European Environmental and Contact Dermatitis Research Group. Risk assessment failures of chemicals commonly used in consumer products. *Contact Dermatitis* 2002: **46**: 189–190.

4 Scientific Committee on Consumer Products (SCCP). Memorandum, classification and categorization of skin sensitizers and grading of test reactions, 20 September 2005, SCCP/0919/05, 2005. Available at: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_s_01.pdf (last accessed 04 November 2014).

5 de Groot A C, Herxheimer A. Isothiazolinone preservative: cause of a continuing epidemic of cosmetic dermatitis. *Lancet* 1989: **1**: 314–316.

6 Dillarstone A. Cosmetic preservatives. *Contact Dermatitis* 1997: **37**: 190.

7 Bruze M, Engfeldt M, Gonçalo M, Goossens A. Recommendation to include methylisothiazolinone in the European baseline patch test series – on behalf of the European Society of Contact Dermatitis and the European Environmental and Contact Dermatitis Research Group. *Contact Dermatitis* 2013: **69**: 263–270.

8 Wilkinson D S, Fregert S, Magnusson B et al. Terminology of contact dermatitis. *Acta Derm Venereol* 1970: **50**: 287–292.

9 CDC (Centers for Disease Control and Prevention), Vaccines and immunizations. glossary, 2013. Available at: http://www.cdc.gov/vaccines/about/terms/glossary.htm#e (last accessed 18 December 2014).

10 Thyssen J P, Engkilde K, Lundov M D, Carlsen B C, Menné T, Johansen J D. Temporal trends of preservative allergy in Denmark (1985–2008). *Contact Dermatitis* 2010: **62**: 102–108.

11 Schnuch A, Lessmann H, Geier J, Uter W. Contact allergy to preservatives. Analysis of IVDK data 1996–2009. *Br J Dermatol* 2011: **164**: 1316–1325.

12 Aerts O, Baeck M, Constandt L et al. The dramatic increase in the rate of methylisothiazolinone contact allergy in Belgium: a multicentre study. *Contact Dermatitis* 2014: **71**: 41–48.

13 Uter W, Geier J, Bauer A, Schnuch A. Risk factors associated with methylisothiazolinone contact sensitization. *Contact Dermatitis* 2013: **69**: 231–238.

14 Lammintausta K, Aalto-Korte K, Ackerman L et al. An epidemic of contact allergy to methylisothiazolinone in Finland. *Contact Dermatitis* 2014: **70**: 184–185.

15 Hosteing S, Meyer N, Waton J et al. Outbreak of contact sensitization to methylisothiazolinone: an analysis of French data from the REVIDAL-GERDA network. *Contact Dermatitis* 2014: **70**: 262–269.

16 Schwensen J F, Menné T, Andersen K E, Sommerlund M, Johansen J D. Occupations at risk of developing contact allergy to isothiazolinones in Danish contact dermatitis patients: results from a Danish multicentre study (2009–2012). *Contact Dermatitis* 2014: **71**: 295–302.

17 Johnston G A, Contributing Members of the British Society for Cutaneous Allergy

(BSCA). The rise in prevalence of contact allergy to methylisothiazolinone in the British Isles. *Contact Dermatitis* 2014: **70**: 238–240.

18 Aerts O, Cattaert N, Lambert J, Goossens A. Airborne and systemic dermatitis, mimicking atopic dermatitis, caused by methylisothiazolinone in a young child. *Contact Dermatitis* 2013: **68**: 250–251.

19 Schwensen J F, Lundov M D, Bossi R et al. Methylisothiazolinone and benzisothiazolinone are widely used in paint: a multicentre study of paints from five European countries. *Contact Dermatitis* 2015: **72**: 127–138.

20 European Parliament and the Council of the European Union. L342/59-209. Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on cosmetic products. *Off J Eur Union* 2009: **L342**. Available at: http://eur-lex.europa.eu/legalcontent/EN/TXT/PDF/?uri=CELEX:32009R1223&from=EN (last accessed 10 November 2014).

21 Gonçalo M, Goossens A. Whilst Rome burns: the epidemic of contact allergy to methylisothiazolinone. *Contact Dermatitis* 2013: **68**: 257–258.

22 Bruze M, Uter W, Gonçalo M, Lepoittevan J-P, Diepgen T, Orton D. Incompetence and failure to regulate methylisothiazolinone. *Contact Dermatitis* 2015: **72**: 353–354.

23 de Groot A C, de Cock P A, Coenraads P J et al. Methyldibromoglutaronitrile is an important contact allergen in The Netherlands. *Contact Dermatitis* 1996: **34**: 118–120.

24 McFadden J P, Ross J S, Jones A B, Rycroft R J, Smith H R, White I R. Increased rate of patch test reactivity to methyldibromo

glutaronitrile. *Contact Dermatitis* 2000: **42**: 54–55.

25 Bruze M, Gruvberger B, Agrup G. Sensitization studies in the guinea pig with the active ingredients of Euxyl K 400. *Contact Dermatitis* 1988: **18**: 37–39.

26 Andersen F. Final report on the safety assessment of methyldibromo glutaronitrile. *J Am Coll Toxicol* 1996: **15**: 140–165.

27 Wahlkvist H, Boman A, Montelius J, Wahlberg J E. Sensitizing potential in mice, guinea pig and man of the preservative Euxyl K 400 and its ingredient methyldibromo glutaronitrile. *Contact Dermatitis* 1999: **41**: 330–338.

28 Scientific Committee on Consumer Products (SCCP). Opinion on Methyldibromo glutaronitrile (Sensitization only) (SCCP/0863/05). COLIPA No. P77. 15 March 2005, 2005. Available at: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_00f.pdf (last accessed 11 November 2014).

29 Scientific Committee on Cosmetic Products and Non-food Products Intended for Consumers (SCCNFP), Opinion on Methyldibromoglutaronitrile (COLIPA 1, P77) Adopted 04 June 2002. SCCNFP/0585/02, final. Available at: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_060.pdf (last accessed 10 October 2014).

30 Friis U F, Menné T, Flyvholm M A et al. Isothiazolinones in commercial products at Danish workplaces. *Contact Dermatitis* 2014: **71**: 65–74.

31 Scientific Committee on Cosmetic Products and Non-food Products Intended for Consumers (SCCNFP), Opinion on Methylisothiazolinone (P94) – Submission, 18 March 2003,

SCCNFP/0625/02. 2003. Available at: http://ec.europa.eu/food/fs/sc/sccp/out_201.pdf (last accessed 26 May 2015)

32 Scientific Committee on Cosmetic Products and Non-food Products Intended for Consumers (SCCNFP), Opinion on Methylisothiazolinone (P94) – Submission, 23 April 2004, SCCNFP/0805/04, 2004. Available at: http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out270_en.pdf (last accessed 26 May 2015).

33 Basketter D A, Gilmoour N J, Wright Z, Walter T, Boman A, Lidén C. Biocides: characterization of the allergenic hazard of methylisothiazolinone. *Cutan Ocul Toxicol* 2003: **22**: 187–199.

34 Scientific Committee on Consumer Safety (SCCS), Opinion on Methylisothiazolinone (P94) – Submission II, 12 December 2013, SCCS/1521/13, revision of 27 March 2014, 2013. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_145.pdf (last accessed 17 November 2014).

35 Gerberick G F, Ryan C A, Kern P S, Schlatter H, Dearman R J, Kimber I et al. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis* 2005: **16**: 157–202.

36 Estrada E, Patlewicz G, Chamberlain M, Basketter D, Larbey S. Computer-aided knowledge generation for understanding skin sensitization mechanisms: the TOPS-MODE approach. *Chem Res Toxicol* 2003: **16**: 1226–1235.

37 Roberts D W. Methylisothiazolinone is categorized as a strong sensitizer in the murine local lymph node assay. *Contact Dermatitis* 2013: **69**: 261–262.

4.2 Methylisothiazolinone and benzisothiazolinone are widely used in paint: a multicentre study of paints from five European countries – Manuscript II

- 71 water-based paints purchased in five European countries were analysed.

- MI was found in 93% (66/71) and the concentration ranged from 0.7 to 180.9 ppm.

- BIT was found in 95.8% (68/71) and the concentration ranged from 0.1 to 462.5 ppm.

- MCI was found in 23.9% (17/71) in relatively small concentrations (0.26–11.4 ppm).

- MI was found in high concentrations across all five countries with no significant difference between the countries.

- The concentration of BIT was particularly high in Denmark and Sweden.

- In general, Swedish paint contained low concentrations of MI and high concentrations of BIT.

- 49.3% (35/71) were labelled with environmental labels, but no difference in the concentration of MI was registered.

- A comprehensive review was conducted of published non-occupational and occupational cases with contact allergy to isothiazolinones due to exposure to paint.

Methylisothiazolinone and benzisothiazolinone are widely used in paint: a multicentre study of paints from five European countries

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Summary

Background. In view of the current epidemic of contact allergy to methylisothiazolinone (MI), it is important to clarify the extent of use of MI and related isothiazolinones in paints currently available for the consumer and worker in Europe.
Objectives. To elucidate the use and concentrations of MI, methylchloroisothiazolinone (MCI) and benzisothiazolinone (BIT) in paints on the European retail market.
Methods. Wall paints (n = 71) were randomly purchased in retail outlets in five European countries. The paints were quantitatively analysed for their contents of MI, MCI and BIT by high-performance liquid chromatography coupled to tandem mass spectrometry.
Results. MI was found in 93.0% (n = 66) of the paints, with concentrations ranging from 0.7 to 180.9 ppm, MCI in 23.9% (n = 17), ranging from 0.26 to 11.4 ppm, and BIT in 95.8% (n = 68), ranging from 0.1 to 462.5 ppm. High concentrations of MI were found in paints from all five countries. Paints purchased in Denmark and Sweden contained especially high concentrations of BIT.
Conclusion. The use of MI across European countries is extensive. In view of the ongoing epidemic of MI contact allergy, an evaluation of the safety of MI in paints is needed.

Key words: benzisothiazolinone; environmental label; methylchloroisothiazolinone; methylisothiazolinone; paint; safety data sheet.

The isothiazolinones methylisothiazolinone (MI, CAS no. 2682-20-4), methylchloroisothiazolinone (MCI, CAS no. 26172-55-4) and benzisothiazolinone (BIT, CAS no 2634-33-5) are antimicrobial agents. The first two are used as preservatives in cosmetic products, and all three

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are used as biocides in chemical products (non-cosmetic products), for example paints (1–3). Isothiazolinones have been used for >30 years, and isothiazolinones have a well-known capacity to induce skin sensitization (4, 5).

The allergenicity of isothiazolinones is exemplified by epidemics of sensitization to isothiazolinones, for example the epidemic of contact allergy to MCI/MI (3:1 fixed combination; CAS no. 55965-84-9) in the early 1980s. Subsequently, restrictions on the use of MCI/MI in cosmetics and their classification as skin sensitizers in chemical products have led to a decreasing prevalence of contact allergy to MCI/MI in several European countries,

and the prevalence of MCI/MI contact allergy had, until recently, stabilized at $\sim 2\%$ (6–9).

In 2000, MI was introduced by industry as a standalone preservative, that is, without MCI, for use in chemical products ('mixtures' according to the CLP regulation) with no upper limit on concentration. In 2005, MI was permitted for use in cosmetic products at a concentration of up to 100 ppm (10). MI was, according to the local lymph node assay, a strong sensitizer (EC3 0.4), but less potent than MCI/MI (11). However, since 2009, the prevalence of contact allergy to MI has increased at an alarming rate throughout Europe (12–19).

The Scientific Committee on Consumer Safety did not consider BIT to be safe for use as a preservative in cosmetic products (20), and so far the prevalence of BIT allergy has remained stable (19). BIT is used as a biocide in a broad range of chemical products (20). Isothiazolinones, especially MI and BIT, are widely used as biocides in paint (2, 3, 21). Moreover, working as a painter has been associated with MI sensitization (15, 22, 23), and several case reports have verified the pattern of paint (both domestic and occupational exposure) being a risk factor for contact allergy to MI.

Isothiazolinones can cause contact allergy by direct skin exposure. As MI is volatile and can therefore evaporate, it may cause airborne allergic contact dermatitis, asthmatic symptoms, and even systemic allergic dermatitis (21, 24–32).

In contrast to the more regulated market for isothiazolinones in cosmetic products, industry can omit warning labelling and information on the use of isothiazolinones in paints. This can be done if the substance is not classified as a skin sensitizer (H317) according to the CLP regulation, or R43 according to its predecessor (the Dangerous Substances Directive), either by legally binding harmonized classification or by notification by industry (also called self-classification). It is problematic for both the consumer and worker with contact allergy to isothiazolinones and for the clinician that product labels and safety data sheets often do not contain information on the isothiazolinone content in the paint, despite a relatively high concentration (33).

In view of the unprecedented epidemic of contact allergy to MI, the presence of isothiazolinones in paints is of particular interest. To our knowledge, the concentrations of isothiazolinones in paints intended for use by the consumer have, on a European basis, not been analysed. The aim of this European multicentre study was to determine the concentrations of MI, MCI and BIT in water-based wall paints purchased in retail outlets for analysis of consumer exposure. Furthermore, a systematic review of the published literature regarding cases with non-occupational and occupational contact allergy to isothiazolinones in paint was conducted.

Materials and Methods

Paint collection and samples

A total of 71 white wall paints or wet room paints were purchased in retail outlets in five European countries: Denmark (Copenhagen), France (Strasbourg), Germany (Erlangen), Sweden (Stockholm), and the United Kingdom (London). The paints were randomly chosen, and represented a broad selection of the brands in each country; all paints were purchased in the period from 1 December 2013 to 31 January 2014. All paints were intended for consumer and/or professional use. Wet room paint was defined as paint intended for use in a humid environment, for example bathrooms.

All paints were sent by post or courier to the Department of Environmental Science, Aarhus University, Denmark, where the cans were opened for the first time and analysed. An attempt was made to buy the same Danish paints as 2 years earlier, but this was not possible (21).

The paint was thoroughly mixed before sampling. If a thin layer of transparent liquid was visible on top in the paint can, a sample was taken before mixing. A portion of ~ 5 ml was taken with a disposable plastic syringe. Analyses were performed in duplicate for randomly chosen samples (every tenth sample).

Safety data sheet and labelling

If possible, safety data sheets were collected for all paints at the time of purchase. If the store did not provide any safety data sheets with the paint, the companies' websites were immediately searched for safety data sheets. All safety data sheets were meticulously searched for warnings and listings of isothiazolinones in the paint.

Additional labelling on the paint cans was also collected. This labelling, however, consisted mainly of environmental labels, for example the European Flower. Environmental labels often have demands regarding the use of isothiazolinones in the paints, and are therefore important for this study. The following environmental and health-related labelling was present on the paint cans: 'EU Ecolabel' ('European Flower'; EU), 'Svane' (The Nordic Swan label; Denmark and Sweden), 'Der Blaue Engel' (The Blue Angel; Germany), 'Svalanmärkt' (Asthma and Allergy Association; Sweden), TÜV NORD: Für Allergiker geeignet, Freiwillige Materialprüfung (optional material testing; recommended for people suffering from allergy) and volatile organic compound (VOC) labelling (low VOC content, minimal VOC content).

The environmental label 'EU Ecolabel' limits isothiazolinones in paints to a total sum of isothiazolinones of 500 ppm, a maximum MI concentration of 200 ppm, a maximum MCI/MI concentration of 15 ppm, and a maximum BIT concentration of 500 ppm (34, 35). The 'Nordic Swan label' (Nordic Ecolabelling) limits isothiazolinones in paints to a total sum of isothiazolinones of 500 ppm, and a maximum MCI/MI concentration of 15 ppm (36). In addition to the environmental label of 'EU Ecolabel', indoor paints can undergo testing for indoor air quality with a specific methodology and, if successful, meet Class A+ ('EU Ecolabel A+'). However 'EU Ecolabel A+' has no additional requirements regarding the use of isothiazolinones in paints (37).

'Der Blaue Engel' limits isothiazolinones in paints to maximum concentrations of 50 ppm MCI/MI, 200 ppm MI, and 200 ppm BIT (38). The 'TÜV NORD' label requires, among other criteria, an MCI/MI concentration of ≤15 ppm, and BIT or BIT and MI concentrations combined of ≤200 ppm (39). Products labelled with 'Svalan' are recommended by the Swedish Asthma and Allergy Association, saying that 'The products are free from allergens, perfumes and irritants in amounts so that no reported medical cases are known' (40). A special provision is given for paints, for which 'the recommendation is valid 2 weeks after application of the paint' (41). VOCs represent a wide variety of compounds, and are used as solvents in paints to help keep the paint stable (37), but the none of the labels with VOC ('Minimal VOC, 0–0.29%'; 'Low VOC, 0.30–7.100%'; 'Medium VOC, 8–24.100%'; High VOC, 25–50%; and 'Very High VOC, more than 50%') have specific requirements regarding the use of isothiazolinone in paints, and are therefore not included as environmental labels of relevance in this analysis (42).

Analysis of isothiazolinones in paint

The concentrations of MI, MCI and BIT were measured in all collected paint samples. As described elsewhere (21), a sample of 1 g ($\pm$ 0.1 g) from each paint was extracted in 25 ml of methanol/0.4% formic acid (20/80 vol/vol) by means of ultrasound over a period of 10 min. The suspension was filtered through a Phenex-GF/CA (fibreglass/cellulose) filter, and analysed by high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry. The analytes were separated on a Kinetex C18 (100×2.1 mm^2) HLPC column, and ionized with electrospray ionization operated in positive mode. The mass spectrometer was operated in multiple reaction monitoring mode, with two mass transitions (parent ion/product ion) for each analyte (m/z 116/101 and

116/71 for MI; m/z 150/87 and 150/135 for MCI; m/z 152/109 and 152/134 for BIT). Detection of the analytes was based on retention time and the most abundant mass transition corresponding to an authentic standard. Confirmation of analyte identity was based on the response of the secondary mass transition relative to the response of the primary mass transition. Quantification of the analytes was performed with response factors calculated from a four-point calibration curve (21).

The recoveries with the extraction method for paint were calculated by spiking five different paints with MI, MCI, and BIT. The samples were spiked at three different concentrations: 0.1, 1.0 or 10 µg/ml. Average recoveries obtained for MI, MCI and BIT were 85.9%, 82.6% and 58.0%, respectively.

The precision of the analysis was calculated as the relative standard deviation of replicate analytes extracted from a total of 12 pairs. The overall precision for MI was 1.3%. The overall precision for BIT was 1.5%.

Review

Literature for a review of non-occupational and occupational cases with contact allergy to isothiazolinones in paint was systematically sought from the PubMed™ database and Google™ scholar. The literature search was carried out with the MeSH terms 'methylisothiazolinone', '2-methyl-4-isothiazolin-3-one', 'methylchloroisothiazolinone', '5-chloro-2-methyl-4-isothiazolin-3-one', 'benzisothiazolinone', '1,2-benzisothiazol-3(2H)-one', Kathon CG', 'CAS no. 26172-55-4', 'CAS no. 2682-20-4', 'CAS no. 2634-33-5', 'CAS no. 55965-84-9', 'contact allergy', 'allergic contact dermatitis', 'airborne', and 'paint'. Reference lists of the relevant articles were also studied for case reports relevant for this review. Only literature in English was included. Overall, case reports were considered for inclusion if contact allergy to isothiazolinone resulting from paint exposure was detected. The last literature search was performed on 1 July 2014.

Statistics

The data were processed with SPSS™ (SPSS™ Statistics Chicago, IL, USA; IBM PASW Statistics) for Windows™, edition 20.0, and R statistical software (version 3.1.0; www.r-project.org).

The Mann−Whitney U-test was applied for (i) analysis of differences between the MI concentrations found in the previously tested Danish paints and the newly found MI contents for Danish paints (21), (ii) analysis of differences between the MI concentrations in paints with environmental labels and paints with no environmental labelling,
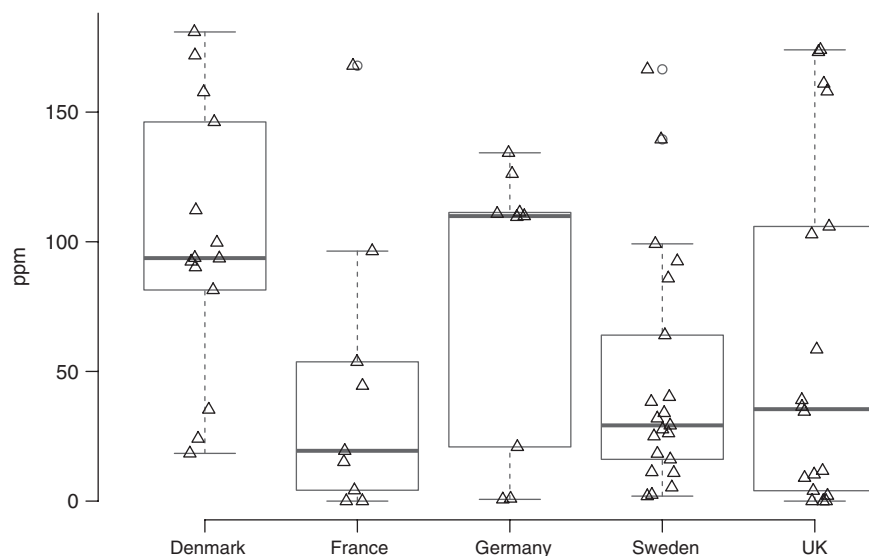
Fig. 1. The distribution of measurements of methylisothiazolinone in wall paints across five European countries, depicted as a boxplot (showing outliers beyond the 1.5-fold interquartile range as dots) with an overlay strip chart, which represents each single measurement as a triangle.

and (iii) analysis of differences between the MI and BIT concentrations in wet room paints and white wall paints. The distribution of the measured values for MI, MCI and BIT were graphically represented by a strip chart with an overlay boxplot. The Kruskal–Wallis *H*-test for global heterogeneity was applied for analysis of differences in MI and BIT concentrations across countries.

The threshold for statistical significance was predefined as a *p*-value of < 0.05.

Results

Seventy-one paints were analysed for their contents of three isothiazolinones. MI was identified in 93.0% (n = 66) of the purchased paints, and the MI concentration ranged from 0.7 to 180.9 ppm (Fig. 1). MCI was identified in 23.9% (n = 17) of the purchased paints, and the MCI concentration ranged from 0.26 to 11.4 ppm. BIT was identified in 95.8% (n = 68) of the purchased paints, and the BIT concentration ranged from 0.1 to 462.5 ppm (Fig. 2).

The distributions of MI concentration differed between countries (Fig. 1). However, no overall statistically significant heterogeneity was seen. In contrast, BIT concentrations differed statistically significantly between countries (Fig. 2).

Table S1 shows a detailed description of all 71 purchased paints regarding product name, MI, MCI and BIT concentrations, and environmental labelling. Seven paints had a low content of MI (< 5 ppm), and five paints (three British and two French) had no detectable MI content at all (Table S1). Only a paint purchased in the United Kingdom contained only MI (4.0 μg/g) with no detectable BIT or MCI. Almost all paints contained MI and BIT in combination. For example, a Danish purchased paint had 180.9 ppm MI in combination with 128.7 ppm BIT. However, many of the Swedish purchased paints contained a relatively high BIT concentration in combination with a relatively low MI concentration, for example 462.5 ppm BIT in combination with 2.47 ppm MI (Table S1).

Samples were also taken from the thin layer of transparent liquid (surface layer) visible on top of nine paints. Analysis showed that the MI concentration in this surface layer was 1.5–2.5 times higher than the MI concentration in the paint in the corresponding can. In a UK paint, an MI concentration of 421.0 ppm was found in the surface layer.

Table 1 shows the frequency of available safety data sheets for all paints in both paint stores and websites for each country. The internet web addresses for the UK purchased paints were not checked immediately after purchase, which is why the data are not included in Table 1. The frequency of environmental labelling is also shown in Table 1. A total of 49.3% (35/71) of the paints were labelled with environmental labels; a Mann–Whitney *U*-test did not reveal any statistically significant difference in MI concentration between the paints with environmental labels and the paints without environmental labels (p = 0.881). All of the UK paints were labelled with either 'VOC Symbol', '99% solvent free', 'Low VOC content 0.3–7.99%', or 'Minimal VOC content 0–0.29%', and, in the analysis, these were not regarded as environmental labels of relevance.

Overall, the labelling of isothiazolinone content on safety data sheets were insufficient for all European

Fig. 2. The distribution of measurements of benzisothiazolinone in wall paints across five European countries, depicted as a boxplot (showing outliers beyond the 1.5-fold interquartile range as dots) with an overlay strip chart, which represents each single measurement as a triangle.
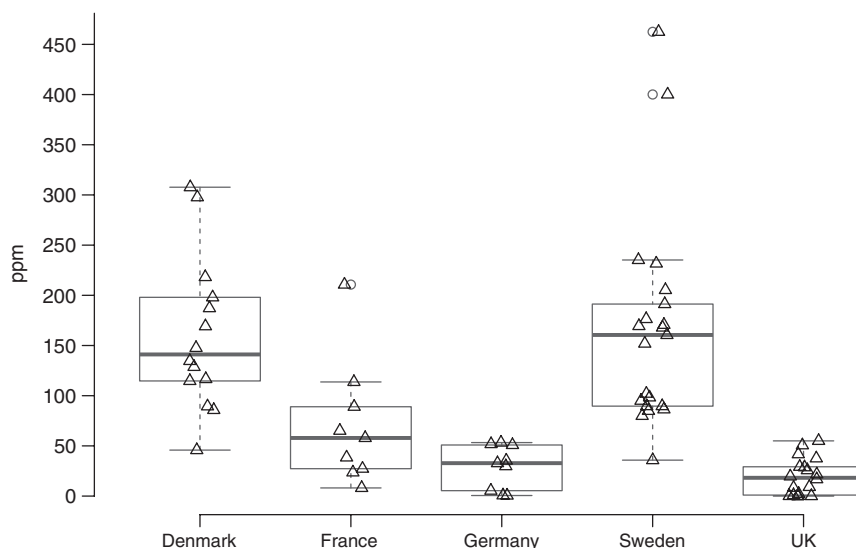
Table 1. Frequency of available safety data sheets (SDSs) for paints at stores or internet websites along with the frequency of paints having environmental labels

European country	SDS at store, % (n/total)	SDS at website, % (n/total)	Environmental labelling on paint cans, % (n/total)
Denmark	0 (0/14)	57.1 (8/14)	64.3 (9/14)
France	0 (0/9)	44.4% (4/9)*	100 (9/9)
Germany	0 (0/9)	88.9 (8/9)*	77.8 (7/9)
Sweden	0 (0/21)	100 (21/21)	42.9 (9/21)
United Kingdom	0 (0/18)	NI	0.0 (0/18)†

NI, not investigated at the time of purchase.

*SDS could be ordered by email.

†Paint cans from the United Kingdom were labelled with VOC (volatile organic compounds). These were not counted as environmental labels. See 'Results'.

countries (Table S1). The manufacturers in the German and Swedish paint markets showed that they were more proactive regarding labelling of isothiazolinone content on the paint cans than manufacturers in other European countries. Two German paints claimed to have no added preservatives on their paint cans. These two paints did indeed have MI and BIT concentrations of < 1 ppm (Table S1).

Furthermore, on a few German paint cans (n = 3), an allergy hotline telephone number was listed in case of allergic symptoms ('Allergiker-Hotline' and 'Technisches Merkblatt').

The Mann–Whitney U-test did not show any statistically significant differences in MI concentration between previously purchased Danish paints in a study by Lundov et al. (21) and the MI concentrations in the Danish paints purchased for the present study ($p = 0.884$).

A total of 19.7% (14/71) paints were wet room paints, and no statistically significant difference in MI concentration between wet room paints and white wall paints was observed ($p = 0.840$), but wet room paints had a statistically significantly higher BIT concentration than white wall paints ($p < 0.001$).

In the past 30 years, several case reports on contact allergy to isothiazolinones resulting from paint exposure have been published (Table 2). Older case reports have primarily presented contact hypersensitivity to MCI/MI (mixture 3:1), BIT (e.g. Proxel™) and octylisothiazolinone in paints (43–50). All of these paints probably contained MCI/MI, BIT, or other isothiazolinones, but in only a few reports were the paints analysed (48). In recent years, after the introduction of MI in 2000, several case reports on contact hypersensitivity to MI or other isothiazolinones resulting from exposure to paint have been published (24–28, 30–32, 51–55). A Danish study described, for the first time, four paint factory workers with MI sensitization and allergic contact dermatitis resulting from direct skin exposure to additives with a 10% MI solution (54). In many of the recently published case reports, allergic contact dermatitis has often developed at directly exposed skin sites, whereas some case reports have shown that emissions of MI can elicit airborne allergic contact dermatitis at indirectly exposed skin sites, for example the face or arms, or even asthmatic symptoms (21, 24–32). Some case reports have described systemic symptoms and generalized dermatitis resulting from exposure to MI and/or BIT in paints (27, 32, 55), and a few studies even reported that emergency treatment was necessary, owing to severe asthmatic symptoms (24, 26).

Table 2. Reports on non-occupational and occupational cases with contact allergy to methylisothiazolinone (MI), benzisothiazolinone (BIT) or methylchloroisothiazolinone (MCI) related to paint exposure

Author		Year of publication	Country	Age (years)	Sex	Occupationally related dermatitis	Positive patch test reactions	Comments	Reference
Mathias		1983	Denmark	31	Male	Yes	OIT (3+)	Contact dermatitis on upper extremity	(43)
Greig		1991	New Zealand	40	Male	Yes	MCI/MI (2+), BIT (2+)	Hand dermatitis after skin exposure to paint and Proxel™ CRL solution containing BIT	(44)
Finkbeiner		1994	Germany	42	Female	No	MCI/MI (1+)	Airborne contact dermatitis (flare-ups). Previously sensitized by cosmetics	(45)
Fernandez de Corrès		1995	Spain	47	Female	Yes (flare-ups)	MCI/MI (2+)	Airborne contact dermatitis (flare-ups). Previously sensitized by cosmetics	(46)
Schubert		1997	Germany	33	Female	Yes (flare-ups)	Unknown (MCI/MI-sensitized)	Airborne contact dermatitis	(47)
Bohn	Patient 1	2000	Switzerland	46	Female	No	MCI/MI (3+)	Airborne contact dermatitis and mild dyspnoea	(48)
	Patient 2			47	Female	No	MCI/MI (3+) patch testing with the paint (3+)	Airborne contact dermatitis with later generalization	
	Patient 3			25	Female	No	MCI/MI	Airborne contact dermatitis, rhinitis, and mild dyspnoea	
	Patient 4			52	Female	No	MCI/MI (2+)	Airborne contact dermatitis, previously sensitized by cosmetics	
	Patient 5			46	Female	No	MCI/MI (2+)	Airborne contact dermatitis	
Hardcastle	Patient 1	2005	United Kingdom	–	Male	Yes	BIT (3+), MCI/MI (1+), OIT (1+?)	Hand dermatitis after skin exposure to paint (paint factory)	(49)
	Patient 2			–	Male	Yes	BIT (1+), MCI/MI (1+), OIT (1+)	Hand dermatitis after skin exposure to paint and later generalization	
Jensen		2006	Germany	13	Male	No	MCI/MI (+1)	Airborne contact dermatitis	(50)
Thyssen	Patient 1	2006	Denmark	55	Male	Yes	MI (2+), MCI/MI (1+)	Hand dermatitis after skin exposure to additives (7–10% MI) at paint factory. Contact dermatitis spread to chest, neck, and armpits	(54)
	Patient 2			40	Male	Yes	MI (1+), OIT (1+), BIT (1+?), MCI/MI (1+?)	Hand dermatitis after skin exposure to additives (7–10% MI) at paint factory	

39

Table 2. Continued

Author		Year of publication	Country	Age (years)	Sex	Occupationally related dermatitis	Positive patch test reactions	Comments	Reference
	Patient 3			34	Male	Yes	MI (2+), MCI/MI (1+?), OIT (1+?)	Hand dermatitis after skin exposure to additives (7–10% MI) at paint factory	
	Patient 4			53	Male	Yes	MI (2+), MCI/MI (2+), OIT (1+?)	Hand dermatitis after skin exposure to additives (7–10% MI) at paint factory. Later generalization	
Garcia-Gavin	Patient 1	2010	Spain/Belgium	55	Male	Yes	1000 ppm MI (3+)	Airborne contact dermatitis and minor dyspnoea (flare-ups). Previously sensitized by wet wipes	(51)
	Patient 2		Spain/Belgium	62	Female	No	MCI/MI (2+) and positive semi-open test result with a piece of Scotex® moist toilet paper (2+)	Airborne contact dermatitis and minor dyspnoea (flare-ups). Previously sensitized by wet wipes and Lactacyd Femina® (MCI/MI content)	
Lundov	Patient 1	2011	Denmark	36	Male	Yes	0.2% MI aqua (1+?)	Airborne contact dermatitis	(26)
	Patient 2		Denmark	57	Male	Unknown	Known MCI/MI and MI	Airborne contact dermatitis and dyspnoea (FEV1 =39%) (flare-ups). Emergency treatment	
Kaur-Knudsen		2012	Denmark	35	Male	Yes	BIT (initially on patch testing) and MCI/MI (later flare-up symptoms)	Airborne contact dermatitis with later generalized dermatitis (flare-ups). Previously sensitized to Proxel™	(55)
Friis		2012	Denmark	64	Male	Yes	MI (2+), MCI/MI (2+)	Contact dermatitis	(52)
Kaae		2012	Denmark	23	Female	Unknown	MCI/MI (2+), MI (2+)	Airborne allergic contact dermatitis. Facial dermatitis when using Nivea® Visage cleaning product (flare-ups)	(28)
Tokunaga		2013	Japan	66	Male	Yes	MCI/MI (1+), BIT (+?)	Airborne allergic contact dermatitis	(29)
Vanneste		2013	Belgium/Sweden	39	Female	No	MI (1+)	Contact dermatitis after skin exposure to paint. Later airborne contact dermatitis caused by paint and cosmetics wet wipes (flare-ups)	(53)

Table 2. Continued

Author	Year of publication	Country	Age (years)	Sex	Occupationally related dermatitis	Positive patch test reactions	Comments	Reference
Aerts	2013	Belgium	4	Girl	No	MCI/MI (1+). No patch testing with MI	Anogenital dermatitis (wet wipes). Airborne contact dermatitis (flare-ups; paint, 53 ppm MI)	(30)
Lundov	2013	Denmark	53	Female	No	MI (1+)	Airborne contact dermatitis, dyspnoea	(31)
Bregnbak	2013	Denmark	42	Female	Yes	MI (1+)	Airborne contact dermatitis	(27)
Bregnbak	2013	Denmark	3	Boy	No	MI (2+), MCI/MI (2+)	Airborne contact dermatitis. Flare-up symptoms when re-exposed to paint, sunscreeen, wet wipes, and shampoo. The contact dermatitis mimicked atopic dermatitis	(32)
Madsen	2014	Denmark	3	Girl	No	MI (3+), MCI/MI (2+)	Airborne contact dermatitis. Previously sensitized to wet wipes	(25)
Alwan	2014	United Kingdom	52	Female	No	MI (3+)	Airborne contact dermatitis and dyspnoea. Emergency treatment	(24)

FEV1, forced expiratory volume in 1 second; OIT, octylisothiazolinone.

Discussion

In this European multicentre study, we investigated the concentrations of MI, MCI and BIT in 71 paints randomly purchased in retail outlets in five European countries: Denmark (Copenhagen), France (Strasbourg), Germany (Erlangen), Sweden (Stockholm), and the United Kingdom (London). MI was found in 93.0% (n = 66) of the paints, BIT was found in 95.8% (n = 68) of the paints, and MCI was found in 23.9% (n = 17) of the paints.

These data indicate that MI and BIT are widely used by the paint industry in relatively high concentrations across the five European countries, indicating a European problem.

In a previous study from Denmark, Lundov et al. found MI concentrations ranging from 10 to 300 ppm in 19 randomly chosen water-based paints purchased in 2012 (21). In the present experimental study, employing a current sample of European paints, the highest MI concentration was found in a Danish purchased paint, with a concentration of 180.9 ppm. In comparison with the previously mentioned study by Lundov et al., it was found that 32% (6/19) of the analysed paints had a higher MI concentration than the highest measured MI concentration of 180.9 ppm, but no statistically significant difference was found (21). However, the MI concentration varies greatly among the Danish purchased paints. Furthermore, the data indicate that the use of MI in paints is a European problem, not being limited to Denmark, and this emphasizes the need for a European evaluation of the health risk caused by MI in paints, and a regulatory limit for MI in paint.

It is likely that paint manufacturers add different isothiazolinones, and probably also other preservatives, to the paint to enhance the antimicrobial effect. By adding different preservatives to the paint, the paint manufacturers also would avoid the need for warning labelling, as the concentrations would be lower than if only a few preservatives were used in high concentrations, for example above 1000 ppm. Our data indicate that more than one isothiazolinone is often added to the paint, as only four paints contained only BIT and only one paint contained only MI. No paints contained only MCI, as expected, as MCI is employed in a fixed 3:1 combination with MI (MCI/MI 3:1). The BIT concentrations in the purchased paints varied among countries. Paints from Denmark and Sweden contained relatively high concentrations of BIT as compared with paints from France, Germany, and the United Kingdom (Fig. 2 and Table S1). Our data indicate that it is possible for the paint manufacturers to preserve paint without the use of a relatively high MI concentration, as some paints contained relatively low MI concentrations, and this was not related to the intended use of the paint,

for example white wall paint versus wet room paint. However, the BIT concentration in paint may be related to the intended use of the paint, as wet room paint had a statistically significantly higher BIT concentration than white wall paint.

In a recent Danish emission test and a field experiment test, it was shown that MI is emitted from newly painted walls within hours, and that a (low) MI concentration is emitted for weeks (21). The published case reports of airborne contact allergy to MI resulting from exposure to paint (24–28, 30–32, 51, 52, 55) are now further supported, as our data indicate that MI is widely used in European paints. In the present study, the MCI concentration in paint was relatively low as compared with the MI and BIT concentrations. MI, as a separately added preservative, had obviously been used additionally to MCI (supposedly Kathon™), as the MI concentration was 2.5–101.3 times higher than the MCI concentration.

Environmental labels often also have provisions for the use of isothiazolinones in the paint; for example, the Environmental label 'EU Ecolabel' limits isothiazolinones in paints (34, 35). None of the paints with the 'EU Ecolabel' contained MI or BIT above the relatively high concentration limits (MI > 200 ppm; BIT > 500 ppm), and the environmental labelling may therefore give the consumer a false sense of security by pretending that the product is safer than the rest of the products. However, our analysis showed no difference in MI concentration between paints labelled with environmental labels and those without additional labelling.

Anti-skinning agents are used to prevent skinning during the production or storage of paints. The thin transparent liquid layer visible on top of some of the paints in the present study was most likely such an anti-skinning agent. Furthermore, the MI concentration in all surface layers was higher than that in the corresponding mixed paint. It is not known to what extent the higher concentration of preservatives in anti-skinning agents adds to the allergy risk.

The current legislation on labelling (CLP) states that chemical products (mixtures) containing a skin sensitizer above a certain concentration should be labelled with a warning to protect against sensitization (56). This is according to the rules of self-classification (notification by industry), if no legally binding (harmonized) classification has been decided. The generic concentration for classification and labelling is 10 000 ppm (1%), but lower, and specific, concentration limits should be set when appropriate. There are 1727 notifications of MI as a skin sensitizer (H317); however, only 52 give a lower specific concentration limit (1000 ppm or 0.1%) (57). The CLP also states that information should be given in safety data sheets and

on the label if the concentration of a classified sensitizer in a product is above one tenth of the concentration limit for classification (called the concentration limit for elicitation).

All analysed paints had MI concentrations well below 1000 ppm and the paint manufacturers were therefore not obliged by law to state that the paints contained MI, despite the risk of contact allergy shown in several studies (Table 2).

For the consumer and the professional decorator, it is currently almost impossible to obtain knowledge about the isothiazolinone content in the paint. Labels and safety data sheets did not generally state the presence of any of the isothiazolinones (Table S1). Safety data sheets were not available at any of the paint stores in the five European cities where the paints were purchased (Table 1). Safety data sheets could, for some of the paints, be obtained at the paint manufacturers' websites, but national differences were observed (Table 1).

The results concerning some paints from Germany and Sweden clearly show that paint manufacturers, regardless of an inadequate European regulation, are able to provide information on isothiazolinone content by labelling and in safety data sheets.

In conclusion, we emphasize an urgent need for evaluation of the regulation on the use of MI in paints for protection of the consumer, the worker, and the MI-allergic patient. It is important for sufficient product labelling of MI content to be made a legal requirement, regardless of the MI concentration. Ultimately, we must emphasize that the paint manufacturers also have a responsibility to improve the safety profile of their paints, for example by stating the use of MI on the paint container, or by limiting, or even abandoning, the use of MI in paints.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Detailed description of all 71 purchased paints regarding product name, product type, concentrations of isothiazolinones, labelling of the presence of isothiazolinone in the paint, and environmental labelling.

References

1 Thyssen J P, Johansen J D, Menné T. Contact allergy epidemics and their controls. *Contact Dermatitis* 2007: **56**: 185–195.

2 Flyvholm M A. Preservatives in registered chemical products. *Contact Dermatitis* 2005: **53**: 27–32.

3 Friis U F, Menné T, Flyvholm M A, Bonde J P, Lepoittevin J P, Le Coz C J, Johansen J D. Isothiazolinones in commercial products at Danish workplaces. *Contact Dermatitis* 2014: **71**: 65–74.

4 Basketter D A, Rodford R, Kimber I, Smith I, Wahlberg J E. Skin sensitization risk assessment: a comparative evaluation of 3 isothiazolinone biocides. *Contact Dermatitis* 1999: **40**: 150–154.

5 Gerberick G F, Ryan C A, Kern P S et al. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. *Dermatitis* 2005: **16**: 157–202.

6 Wilkinson J D, Shaw S, Andersen K E et al. Monitoring levels of preservative sensitivity in Europe. A 10-year overview (1991–2000). *Contact Dermatitis* 2002: **46**: 207–210.

7 Thyssen J P, Engkilde K, Lundov M D, Carlsen B C, Menné T, Johansen J D. Temporal trends of preservative allergy in Denmark (1985–2008). *Contact Dermatitis* 2010: **62**: 102–108.

8 Scientific Committee on Consumer Safety. Opinion on the mixture of 5-chloro-2-methylisothiazolin-3(2H)-one and 2-methylisothiazolin-3(2H)-one, Colipa no P56, 2009. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_009.pdf (last accessed 02 May 2014).

9 Geier J, Lessmann H, Schnuch A, Uter W. Recent increase in allergic reactions to methylchloroisothiazolinone/methylisothiazolinone: is methylisothiazolinone the culprit? *Contact Dermatitis* 2012: **67**: 334–341.

10 SCCS. Scientific Committee on Consumer Safety. Opinion on methylisothiazolinone (P94) Submission II (sensitisation only). Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_145.pdf. (last accessed 17 November 2014)

11 Basketter D A, Gilmour N J, Wright Z M, Walters T, Boman A, Lidén C. Biocides: characterization of the allergenic hazard of methylisothiazolinone. *Cutan Ocul Toxicol* 2003: **22**: 187–199.

12 Lundov M D, Thyssen J P, Zachariae C, Johansen J D. Prevalence and cause of methylisothiazolinone contact allergy. *Contact Dermatitis* 2010: **63**: 164–167.

13 Gonçalo M, Goossens A. Whilst Rome burns: the epidemic of contact allergy to methylisothiazolinone. *Contact Dermatitis* 2013: **68**: 257–258.

14 Urwin R, Wilkinson M. Methylchloroisothiazolinone and methylisothiazolinone contact allergy: a new 'epidemic'. *Contact Dermatitis* 2013: **68**: 253–255.

15 Uter W, Geier J, Bauer A, Schnuch A. Risk factors associated with methylisothiazolinone contact sensitization. *Contact Dermatitis* 2013: **69**: 231–238.

16 Lammintausta K, Aalto-Korte K, Ackerman L et al. An epidemic of contact allergy to methylisothiazolinone in Finland. *Contact Dermatitis* 2014: **70**: 184–185.

17 Hosteing S, Meyer N, Waton J et al. Outbreak of contact sensitization to methylisothiazolinone: an analysis of French data from the REVIDAL-GERDA network. *Contact Dermatitis* 2014: **70**: 262–269.

18 McFadden J P, Mann J, White J M, Banerjee P, White I R. Outbreak of methylisothiazolinone allergy targeting those aged ≥ 40 years. *Contact Dermatitis* 2013: **69**: 53–55.

19 Lundov M D, Opstrup M S, Johansen J D. Methylisothiazolinone contact allergy – growing epidemic. *Contact Dermatitis* 2013: **69**: 271–275.

20 Scientific Committee on Consumer Safety. Opinion on benzisothiazolinone. European Commission Health & Consumers, 2012. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_099.pdf (last accessed 02 May 2014).

21 Lundov M D, Kolarik B, Bossi R, Gunnarsen L, Johansen J D. Emission of isothiazolinones from water-based paints. *Environ Sci Technol* 2014: **48**: 6989–6994.

22 Mose A P, Lundov M D, Zachariae C et al. Occupational contact dermatitis in painters: an analysis of patch test data from the Danish Contact Dermatitis Group. *Contact Dermatitis* 2012: **67**: 293–297.

23 Schwensen J F, Menné T, Andersen K E, Sommerlund M, Johansen J D. Occupations at risk for developing contact allergy to isothiazolinones in Danish contact dermatitis patients: results from a Danish Multicentre study (2009–2012). *Contact Dermatitis* 2014: **71**: 295–302.

24 Alwan W, White I R, Banerjee P. Presumed airborne contact allergy to methylisothiazolinone causing acute severe facial dermatitis and respiratory difficulty. *Contact Dermatitis* 2014: **70**: 320–321.

25 Madsen J T, Andersen K E. Airborne allergic contact dermatitis caused by methylisothiazolinone in a child sensitized from wet wipes. *Contact Dermatitis* 2014: **70**: 183–184.

26 Lundov M D, Mosbech H, Thyssen J P, Menné T, Zachariae C. Two cases of airborne allergic contact dermatitis caused by methylisothiazolinone in paint. *Contact Dermatitis* 2011: **65**: 176–179.

27 Bregnbak D, Lundov M D, Zachariae C, Menné T, Johansen J D. Five cases of severe chronic dermatitis caused by isothiazolinones. *Contact Dermatitis* 2013: **69**: 57–59.

28 Kaae J, Menné T, Thyssen J P. Presumed primary contact sensitization to methylisothiazolinone from paint: a chemical that became airborne. *Contact Dermatitis* 2012: **66**: 341–342.

29 Tokunaga M, Fujii H, Okada K, Kagemoto Y, Nomura T, Tanioka M, Matsumura Y, Miyachi Y. Occupational airborne contact dermatitis by isothiazolinones contained in wall paint products. *Allergol Int* 2013: **62**: 395–397.

30 Aerts O, Cattaert N, Lambert J, Goossens A. Airborne and systemic dermatitis, mimicking atopic dermatitis, caused by methylisothiazolinone in a young child. *Contact Dermatitis* 2013: **68**: 250–251.

31 Lundov M D, Friis U F, Menné T, Johansen J D. Methylisothiazolinone in paint forces a patient out of her apartment. *Contact Dermatitis* 2013: **69**: 252–253.

32 Bregnbak D, Johansen J D. Airborne sensitization to isothiazolinones observed in a 3-month-old boy. *Contact Dermatitis* 2013: **69**: 55–56.

33 Friis U F, Menné T, Flyvholm M A, Bonde J P, Johansen J D. Occupational allergic contact dermatitis diagnosed by a systematic stepwise exposure assessment of allergens in the work environment. *Contact Dermatitis* 2013: **69**: 153–163.

34 The European Commission. Commission decision of 28 May 2014 establishing the ecological criteria for the award of the EU Ecolabel for indoor and outdoor paints and varnishes (notified under document C(2014) 3429) (2014/312/EU). *Off J Eur Union* 2014: L164: 45–73. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32014D0312&from=EN (last accessed 22 July 2014).

35 European Commission Ecolabel criteria and assessment and verification requirements (D0229991/02). Available at: http://ec.europa.eu/transparency/regcomitology/index.cfm?do=Search.getPDF&VVNozgoQrJF+hNKwYYS948a7UWnazQGdiMBzoap7tlWyEZEdggz+n4JOVUqYppXe7kGvLzo2Pu5uyjPyPE0HGhn1Yyu8a5hceFqN5ixnqYI= (last accessed 17 November 2014).

36 Nordic Ecolabelling of Indoor paints and varnishes. Version 2.4, 4 November 2008–30 June 2016. 19 March 2014. Available at: http://www.nordic-ecolabel.org/Templates/Pages/CriteriaPages/CriteriaGetFile.aspx?fileID=170394001 (last accessed 08 September 2014).

37 Joint Research Centre (JRC). Revision of EU European Ecolabel for Indoor and Outdoor paints and varnishes. EU Ecolabel Criteria Proposal Report, 2012. Available at: susproc.jrc.ec.europa.eu/paints/docs/Ecolabel Paints Criteria_for website.pdf (last accessed 05 August 2014).

38 CHEMIE.DE Information Service GmbH. Available at: www.chemie.de/lexikon/Isothiazolinones.html (last accessed 29 August 2014).

39 Fraunhofer WKI. Vergabekriterien 'Schadstoffgeprüft'. Produktgruppe 'Dispersionsinnenfarben'. WKI Prüfspezifikation. WKI-PS-DIF-002, Version 1.2. Available at: http://www.tuev-nord.de/cps/rde/xbcr/SID-036F175A-F432A473/tng_de/dispersionsinnen

farben-kriterien-wki.pdf (last accessed 29 August 2014).

40 Astma och Allergi Förbundet. Policy for advisory review board, 2008. Available at: http://astmaoallergiforbundet.se/svalanmarkt/ (last accessed 20 September 2014).

41 Astma och Allergi Förbundet. Rekommenderade produkter. Målarfärger. Available at: http://astmaoallergiforbundet.se/svalanmarkt/malarfarger/ (last accessed 20 September 2014).

42 British Coating Federation (BCF). Environmental Claims – lead. VOC labelling scheme for decorative coatings. Available at: http://www.coatings.org.uk/BCF_Matters/Environmental_Claims_lead.aspx (last accessed 05 August 2014).

43 Mathias C G, Andersen K E, Hamann K. Allergic contact dermatitis from 2-n-octyl-4-isothiazolin-3-one, a paint mildewcide. *Contact Dermatitis* 1983: **9**: 507–509.

44 Greig D E. Another isothiazolinone source. *Contact Dermatitis* 1991: **25**: 201–202.

45 Fenkbeiner H, Kleinhans D. Airborne allergic contact dermatitis caused by preservatives in home-decorating paints. *Contact Dermatitis* 1994: **31**: 275–276.

46 Fernández de Corrés L, Navarro J A, Gastaminza G, Del Pozo M D. An unusual case of sensitization to methylchloro- and methyl-isothiazolinone (MCI-MI). *Contact Dermatitis* 1995: **33**: 215–216.

47 Schubert H. Airborne contact dermatitis due to methylchloro- and methylisothiazolinone (MCI/MI). *Contact Dermatitis* 1997: **36**: 274.

48 Bohn S, Niederer M, Brehm K, Bircher A J. Airborne contact dermatitis from methylchloroisothiazolinone in wall paint. Abolition of symptoms by chemical allergen inactivation. *Contact Dermatitis* 2000: **42**: 196–201.

49 Hardcastle N J, Gawkrodger DJ. Occupational contact dermatitis to 2-benzisothiazolin-3-one and 5-chloro-2-methylisothiazolin-3-one/2-methylisothiazolin-3-one in paint manufactures. *Contact Dermatitis* 2005: **53**: 115–116.

50 Jensen J M, Harde V, Brasch J. Airborne contact dermatitis to methylchloroisothiazolinone/methylisothiazolinone in a boy. *Contact Dermatitis* 2006: **55**: 311.

51 Garcia-Gavin J, Vansina S, Kerre S, Naert A, Goossens A. Methylisothiazolinone, an emerging allergen in cosmetics? *Contact Dermatitis* 2010: **63**: 96–101.

52 Friis U F, Menné T, Thyssen J P, Johansen J D. A patient's drawing helped the physician to make the correct diagnosis: occupational contact allergy to

45

isothiazolinone. *Contact Dermatitis* 2012: **67**: 174–176.

53 Vanneste L, Persson L, Zimerson E, Bruze M, Luyckx R, Goossens A. Allergic contact dermatitis caused by methylisothiazolinone from different sources, including 'mislabelled' household wet wipes. *Contact Dermatitis* 2013: **69**: 311–312.

54 Thyssen J P, Sederberg-Olsen N, Thomsen J F, Menné T. Contact dermatitis from methylisothiazolinone in a paint factory. *Contact Dermatitis* 2006: **54**: 322–324.

55 Kaur-Knudsen D, Menné T, Christina Carlsen B. Systemic allergic dermatitis following airborne exposure to 1,2-benzisothiazolin-3-one. *Contact Dermatitis* 2012: **67**: 310–312.

56 European Chemicals Agency (ECHA). Guidance on the application of the CLP criteria. Guidance to Regulation (EC) No. 1272/2008 on classification, labelling and packaging (CLP) of substances and mixtures. Version 4.0, November 2013. Available at: echa.europa.eu/ documents/10162/13562/clp_en.pdf (last accessed 22 July 2014).

57 European Chemicals Agency (ECHA). C&L inventory database, Last updated 4 July 2014. Available at: http://echa.europa. eu/web/guest/information-on-chemicals/ cl-inventory-database (last accessed 22 July 2014).

4.3 Cross-reactivity between methylisothiazolinone, octylisothiazolinone and benzisothiazolinone using a modified local lymph node assay – Manuscript III

- No significant impurities of isothiazolinones in other isothiazolinone standards were found.
- MI induced strong concentration-dependent immune responses in the draining lymph nodes after a sensitisation phase of three consecutive days.
    - Overall, the test for global heterogeneity for ear swelling, CD4$^+$ BrdU$^+$ T cells, CD8$^+$ BrdU$^+$ T cells, and CD19$^+$ BrdU$^+$ B cells was statistically significant. However, post-hoc pair-wise comparisons showed in general terms only a partial statistical significance; some pair-wise comparisons were not statistically significant.
- The challenge experiments showed that MI-sensitised mice irrespective of being exposed to 0.4% MI, 0.7% OIT or 1.9% BIT reacted equally with ear swelling and showed a similar immune response regarding CD4$^+$ BrdU$^+$ T cells in the draining auricular lymph nodes.
- The challenge experiments also showed that MI-sensitised mice irrespective of being exposed to 0.4% MI, 0.7% OIT or 1.9% BIT activated the immune response similarly regarding CD8$^+$ BrdU$^+$ T cells and partly for CD19$^+$ BrdU$^+$ B cells.

Cross-reactivity between methylisothiazolinone, octylisothiazolinone and benzisothiazolinone using a modified local lymph node assay*

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Summary

Background In the light of the exceptionally high rates of contact allergy to the preservative methylisothiazolinone (MI), information about cross-reactivity between MI, octylisothiazolinone (OIT) and benzisothiazolinone (BIT) is needed.
Objectives To study cross-reactivity between MI and OIT, and between MI and BIT.
Methods Immune responses to MI, OIT and BIT were studied in vehicle and MI-sensitized female CBA mice by a modified local lymph node assay. The inflammatory response was measured by ear thickness, cell proliferation of $CD4^+$ and $CD8^+$ T cells, and $CD19^+$ B cells in the auricular draining lymph nodes.
Results MI induced significant, strong, concentration-dependent immune responses in the draining lymph nodes following a sensitization phase of three consecutive days. Groups of MI-sensitized mice were challenged on day 23 with 0·4% MI, 0·7% OIT and 1·9% BIT – concentrations corresponding to their individual EC3 values. No statistically significant difference in proliferation of $CD4^+$ and $CD8^+$ T cells was observed between mice challenged with MI compared with mice challenged with BIT and OIT.
Conclusions The data indicate cross-reactivity between MI, OIT and BIT, when the potency of the chemical was taken into account in choice of challenge concentration. This means that MI-sensitized individuals may react to OIT and BIT if exposed to sufficient concentrations.

> ## What's already known about this topic?
>
> - Contact allergy to methylisothiazolinone (MI) in the European population is alarmingly high.
> - Retrospective observational studies of patients with contact allergy to MI have shown that concomitant reactions between MI, octylisothiazolinone (OIT) and benzisothiazolinone (BIT) may exist.

> ## What does this study add?
>
> - MI induced a significant concentration-dependent immune response after a sensitization phase of three consecutive days.
> - MI, OIT and BIT induced the same concentration-dependent inflammatory response with proliferation of $CD4^+$ and $CD8^+$ T cells, and partly $CD19^+$ B cells, in MI-sensitized mice.
> - Cross-reactivity was seen between MI and OIT and between MI and BIT when the potency of the chemical was taken into account in the choice of challenge concentration.

The introduction of the preservative methylisothiazolinone (MI) in cosmetic products on the European market has resulted in an unprecedented epidemic of contact allergy and allergic contact dermatitis to MI.[1–4] Currently, 100 ppm (0·01%) MI is allowed in cosmetic products in the European Union. However, the recent European risk management assessment of MI in cosmetic products, which was performed on the basis of newly achieved evidence showing that MI possesses a greater sensitizing potential than originally anticipated, has resulted in the publication of new recommendations on the use of MI in cosmetic products.[5–8] It is therefore important to obtain sufficient knowledge on the potential cross-reactivity between MI and other common isothiazolinones, as the cosmetic industry is eager to substitute MI with other isothiazolinones, for example benzisothiazolinone (BIT).[9]

The chemical structures of MI, octylisothiazolinone (OIT) and BIT are similar; they all contain an isothiazolinone ring (Fig. 1), which may indicate that cross-reactivity exists between these isothiazolinones. The only systematic study testing cross-reactivity between MI and methylchloroisothiazolinone (MCI) was conducted by Bruze *et al.* in the 1980s, using the guinea pig maximization test.[10] Interestingly, several observational studies have described potential cross-reactivity between selected isothiazolinones, but it is currently unknown to what extent cross-reactivity exists in patients with MI contact allergy.[1,11–15]

In general, and in a historical context, animal studies have proven useful in estimating the allergenic capacity of a chemical substance and the cross-reactivity between structurally related chemicals.[16] Further studies on isothiazolinones and their cross-reactivity are needed to (i) ensure that patients with a newly diagnosed MI contact allergy receive the best possible medical advice; and to (ii) give legislative authorities the basis to regulate sufficiently the use of isothiazolinones in products. The aim of this study was to investigate whether MI-sensitized mice developed the same immune response when being challenged with MI as with OIT and BIT.

Materials and methods

Mice

Female CBA mice were purchased from Janvier Labs (Saint-Berthevin, France). All mice were housed in the specific pathogen-free animal facility of the University of Copenhagen

in accordance with national animal protection guidelines (licence number 2012-15-2934-00663). All mice were acclimatized for 1 week before the experiments started at the age of 7–8 weeks. All mice were housed in conventional filter-top cages with standardized light/dark cycles. All mice received water and pelleted food *ad libitum*.

Isothiazolinones

The following chemicals and isothiazolinones were purchased from Sigma-Aldrich (St Louis, MO, U.S.A.) between September 2014 and January 2015: acetone (CAS-RN:67-64-1); olive oil (CAS RN: 8001-25-0); MI (CAS RN: 2682-20-4); octylisothiazolinone (CAS RN 26530-20-1); BIT (CAS RN: 2634-33-5).

EC3 values

The sensitizing hazard for contact allergens can be quantified by derivation of the EC3 value, which is estimated as the allergen concentration necessary to induce a threefold increase in proliferation activity in the draining lymph node.[17]

The challenge concentrations were chosen on the basis of published EC3 values: 0·4% for MI and 1·9% for BIT.[18–20] No EC3 value for OIT could be identified in the literature.[21] We therefore estimated the EC3 value of OIT based on the fact that, chemically, OIT is a homologue of MI (Fig. 1). The reaction chemistry of OIT compared with MI is either the same or possibly less reactive because of the steric effect of the octyl group in the OIT molecule (Fig. 1).

For this murine study, OIT was regarded as a strong sensitizer with an estimated EC3 value of 0·7% as we assumed that for MI and its homologues the potency is not logP dependent. Molar potency would then be the same, but in terms of weight percentage it would depend on molecular weight, owing to the octyl homologue (Fig. 1). On the basis of the EC3 value of MI, we multiplied by the ratio of the molecular weights 213/115 to get and EC3 value for OIT of 0·7%.

Purity analysis of the used standards

In order to exclude cross-contamination from other isothiazolinones, purity analyses of each used standard were performed. Stock solutions (0·2 mg mL^{-1}) were prepared in methanol (Merck, Darmstadt, Germany). The analysis was performed on an Ultimate 3000 dual-gradient, low-pressure mixing high-performance liquid chromatography system (Dionex, Sunnyvale, CA, U.S.A.) coupled to an API 4000 triple-quadrupole mass spectrometer (AB Sciex, Framingham, MA, U.S.A.) with electrospray ionization in positive mode.[22] Each isothiazolinone was analysed on two precursor/product–ion pairs. Limits of detection ranged from 0·06 to 0·5 ng mL^{-1}. None of the isothiazolinones could be detected in any of the other standards. Hence, impurities of < 0·03% were calculated based on stock solution concentration and detection limit (see Table 1).
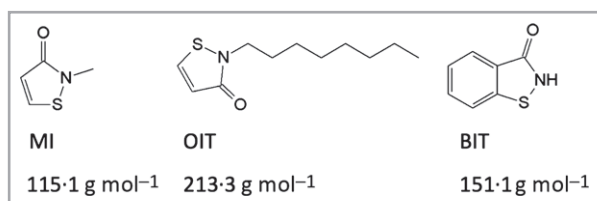

Fig 1. Chemical structures and molar masses of methylisothiazolinone (MI), octylisothiazolinone (OIT) and benzisothiazolinone (BIT).

Table 1 Impurities of isothiazolinones in other isothiazolinone standards

	Impurities (%)		
Standard	Methylisothiazolinone	Benzisothiazolinone	Octylisothiazolinone
Methylisothiazolinone	–	< 0·01	< 0·003
Benzisothiazolinone	< 0·03	–	< 0·005
Octylisothiazolinone	< 0·02	< 0·02	–

Induction of contact sensitization

To induce contact hypersensitivity, each mouse was exposed to 25 μL newly dissolved MI in a 1 : 4 olive oil:acetone mixture (OOA) on the dorsal side of both ears for three consecutive days (days 0–2). Concentrations were 0·13% MI, 0·4% MI or 1·2% MI. Control mice were exposed to 25 μL vehicle (OOA). All mice were given 0·8 mg mL^{-1} 5-bromo-2′-deoxyuridine (BrdU) in their drinking water on day 3 and euthanized 48 h after, on day 5. Subsequently, skin inflammation of the ears was quantified by the increase in the thickness of the ears, as measured using an engineer's micrometre (Mitutoyo, Kawasaki, Japan). The draining retroauricular lymph nodes were surgically removed for subsequent flow cytometric analysis. After removal, the retroauricular lymph nodes were kept on ice in complete RPMI medium.

Challenge experiments

Mice were exposed to 25 μL newly dissolved mixtures of MI (0·13%, 0·4% or 1·2%) or 25 μL vehicle (OOA; control mice) on the dorsal side of both ears for three consecutive days (days 0–2). All mice were given 0·8 mg mL^{-1} BrdU in their drinking water on day 22, and on day 23 mice were challenged on the dorsal side of both ears with either 25 μL 0·4% MI, 1·9% BIT or 0·7% OIT. Control mice were exposed to 25 μL vehicle or dissolved mixture of 0·4% MI on the dorsal side of both ears. All mice were euthanized 24 h after the day of challenge. Skin inflammation of the ears was quantified by the increase in the thickness of the ears as described above. Additionally, the draining retroauricular lymph nodes were surgically removed for flow cytometry. After removal, the retroauricular lymph nodes were kept on ice in complete RPMI medium.

Flow cytometry

A suspension of cells of the removed draining retroauricular lymph nodes was manually prepared by pressing the lymph nodes through a cell strainer followed by washing in complete RPMI medium. Cells were counted with a haemocytometer and resuspended (10^7 cells mL^{-1}) in complete RPMI medium. The distribution of B and T cells was analysed by incubation with anti-CD4, anti-CD8 and anti-CD19. Cells were stained intracellularly with anti-BrdU to determine cellular proliferation, as previously described.[16] Finally, cells were analysed by flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA, U.S.A.).

Statistics

The data were processed with R (version 3.1.0; www.r-project.org).

All data were normally distributed after log-transformation visually accessed by histograms. A strip chart plot showed the distribution of ear thickness, CD4$^+$ BrdU$^+$ T cells, CD8$^+$ BrdU$^+$ T cells and CD19$^+$ BrdU$^+$ B cells. Preselected one-way ANOVA with post hoc Tukey's honest significant difference (HSD) test for global heterogeneity was applied for analysis of differences in means across subgroups (n = 8). The threshold for statistical significance was predefined as a P-value < 0·05.

Results

Sensitization to methylisothiazolinone

Application of MI at concentrations of 0·13% MI, 0·4% MI or 1·2% MI for three consecutive days induced statistically significant ear swelling (P < 0·001) (Fig. 2a). Further, MI induced significant concentration-dependent immune responses in the draining lymph nodes after the sensitization phase of three consecutive days. The overall test for global heterogeneity for CD4$^+$ BrdU$^+$ T cells, CD8$^+$ BrdU$^+$ T cells and CD19$^+$ BrdU$^+$ B cells assessed by one-way ANOVA was statistically significant (P < 0·001, P < 0·001 and P < 0·05, respectively) (Fig. 2b–d). In addition, pair-wise comparisons, not shown in Figure 2a, indicated that local ear swelling showed a significant concentration-dependent trend for sensitization with MI [induction with 0·13% MI vs. 0·4% MI (P < 0·01), and 0·13% MI and 0·4% MI vs. 1·2% MI (both P < 0·001)]. Further pair-wise comparisons of the immune response with regard to CD4$^+$ BrdU$^+$ T cells showed a partly significant concentration-dependent trend (Fig. 2b): induction with 0·13% MI vs. 0·4% MI (P > 0·05), and 0·13% MI and 0·4% MI vs. 1·2% MI (P < 0·01 and P < 0·001, respectively). The same pair-wise comparisons showed a nonsignificant trend for the immune response with regard to CD8$^+$ BrdU$^+$ T cells and CD19$^+$ BrdU$^+$ B cells.

Challenge experiment: response to methylisothiazolinone, octylisothiazolinone and benzisothiazolinone in methylisothiazolinone-sensitized mice

Figure 3a shows the response, signified by local swelling of the ears, after challenge at day 23 with MI, OIT or BIT. Here,
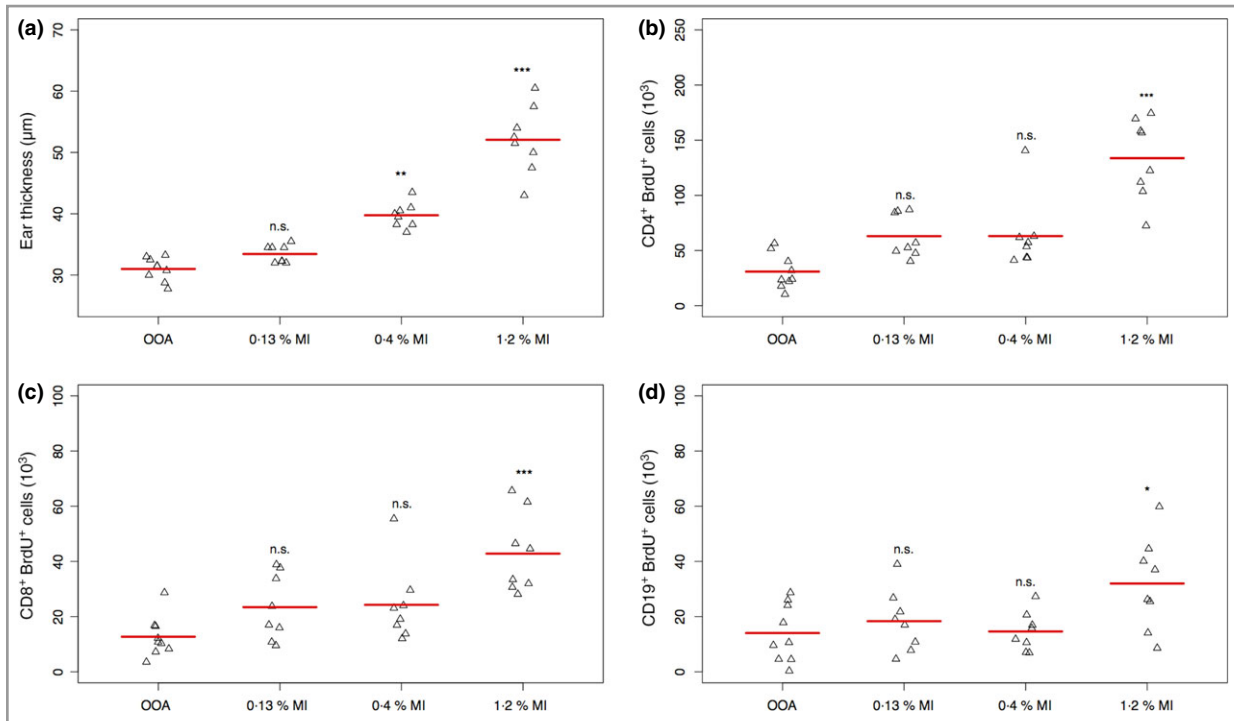
Fig 2. (a) The distribution of ear swelling, (b) $CD4^+$ 5-bromo-2′-deoxyuridine (BrdU)$^+$ T cells, (c) $CD8^+$ BrdU$^+$ T cells and (d) $CD19^+$ BrdU$^+$ B cells in mice sensitized with vehicle, 0·13% methylisothiazolinone (MI), 0·4% MI or 1·2% MI depicted as a strip chart with mean. Each triangle represents a single measurement. The overall test for global heterogeneity for ear thickness, $CD4^+$ BrdU$^+$ T cells, $CD8^+$ BrdU$^+$ T cells and $CD19^+$ BrdU$^+$ B cells assessed by one-way ANOVA were statistically significant. Asterisks signify the outcome of post hoc Tukey's honest significant difference test for the specific subgroup in comparison with the control group. OAA, olive oil and acetone; n.s., nonsignificant ($P > 0.05$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ ($n = 8–9$ based on two independent experiments).

significant differences between the subgroups were observed ($P < 0.001$). All subgroups except mice sensitized with OOA or 0·13% MI and challenged with 0·4% MI showed a statistically significant difference in ear swelling compared with control mice.

Additional pair-wise comparisons found that local swelling of the ears showed a significant concentration-dependent trend when challenged with MI: 0·13% MI vs. 0·4% MI ($P < 0.05$), and 0·13% MI and 0·4% MI vs. 1·2% MI ($P < 0.001$ and $P > 0.05$, respectively). The same pair-wise comparisons were partly significant for challenge with OIT: 0·13% MI vs. 0·4% MI ($P > 0.05$), and 0·13% MI and 0·4% vs. 1·2% ($P < 0.001$ and $P < 0.001$, respectively). Additionally, the pair-wise comparisons were partly significant for BIT: 0·13% MI vs. 0·4% MI ($P > 0.05$), and 0·13% MI and 0·4% vs. 1·2% ($P < 0.001$ and $P < 0.01$, respectively).

Challenge experiment: methylisothiazolinone, octylisothiazolinone and benzisothiazolinone equally activate $CD4^+$ T cells in methylisothiazolinone-sensitized mice

The immune responses in regard to $CD4^+$ T cells in the draining auricular lymph nodes after challenge with MI, OIT and BIT were statistically significant ($P < 0.001$) (Fig. 3b). Mice

sensitized with OOA or 0·13% MI and challenged with 0·4% MI did not show a significant statistic difference compared with control mice. However, MI-sensitized mice challenged with MI, OIT or BIT showed similar, statistically significant $CD4^+$ T-cell proliferation compared with control mice (Fig. 3b). Further pair-wise comparisons of the immune response in regard to $CD4^+$ BrdU$^+$ T cells showed a nonsignificant concentration-dependent trend at the time of sensitization when challenged with MI, OIT and BIT.

Challenge experiment: methylisothiazolinone, octylisothiazolinone and benzisothiazolinone partly activate $CD8^+$ T cells in methylisothiazolinone-sensitized mice

Again, the immune responses in regard to $CD8^+$ T cells in the draining auricular lymph nodes after challenge phases with MI, OIT and BIT were statistically significant ($P < 0.001$; Fig. 3c). Post hoc analyses with Tukey's HSD test comparing the subgroups with control mice showed that only mice sensitized with 1·2% MI showed a statistically significant difference (Fig. 3c).

Additional pair-wise comparisons of the subgroups testing concentration dependency of $CD8^+$ BrdU$^+$ T cells showed, in part, a nonsignificant trend at the time of sensitization when challenged MI, OIT and BIT (data not shown).
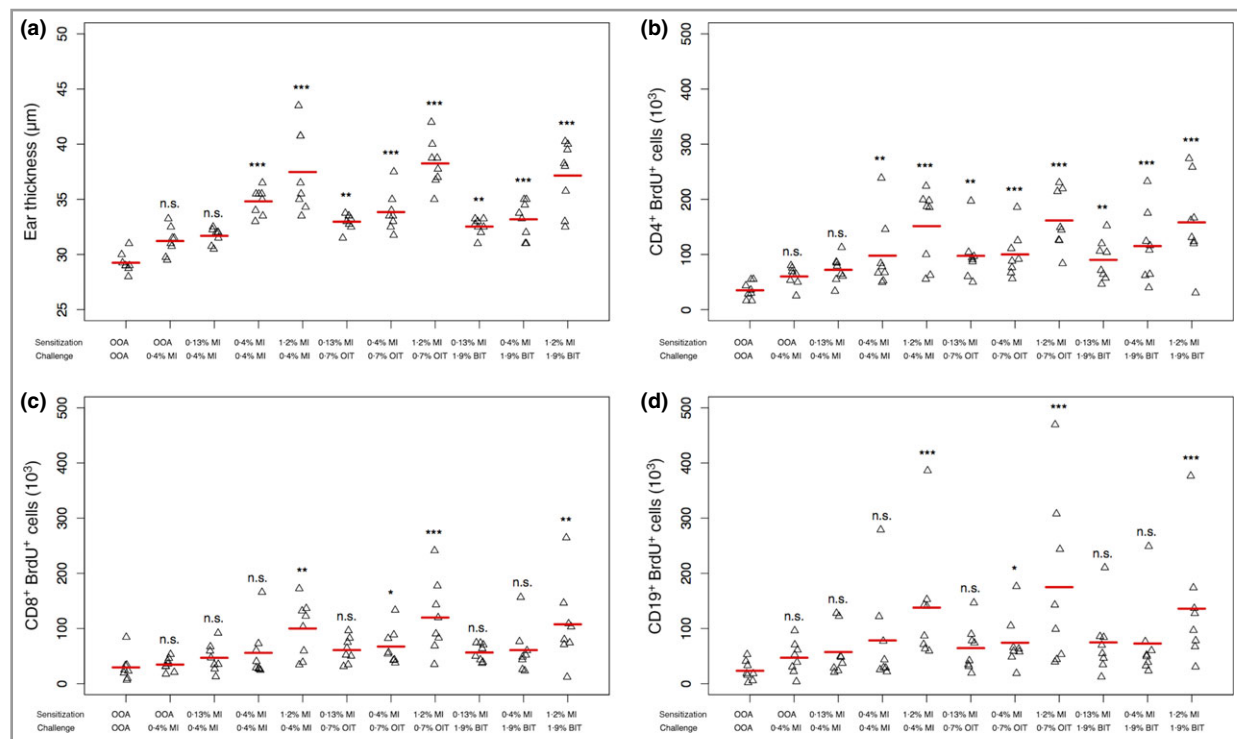
Fig 3. (a) The distribution of ear swelling, (b) CD4^{+} 5-bromo-2′-deoxyuridine (BrdU)[+] T cells, (c) CD8^{+}BrdU^{+} T cells and (d) CD19^{+} BrdU^{+} B cells in mice sensitized with vehicle [olive oil and acetone (OOA)], 0·13% methylisothiazolinone (MI), 0·4% MI or 1·2% MI, and challenged with either vehicle (OOA), 0·4% MI, 0·7% octylisothiazolinone (OIT) or 1·9% benzisothiazolinone (BIT), depicted as a strip chart with mean. Each triangle represents a single measurement. The overall test for global heterogeneity for ear thickness accessed by one-way ANOVA was statistically significant. Asterisks signify the outcome of post hoc Tukey's honest significant difference test for the specific subgroup in comparison with the control group. n.s., nonsignificant ($P > 0.05$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (n = 8 based on two independent experiments).

Challenge experiment: methylisothiazolinone, octylisothiazolinone and benzisothiazolinone partly activate CD19^{+} B cells in methylisothiazolinone-sensitized mice

The immune responses in regard to CD19^{+} BrdU^{+} B cells in the draining auricular lymph nodes after challenge with MI, OIT or BIT were statistically significant ($P < 0.001$; Fig. 3d). Post hoc analyses with Tukey's HSD test comparing the subgroups with control mice showed that only mice sensitized with either 0·4% MI or 1·2% MI and challenged with 0·4% MI, 0·7% OIT or 1·9% BIT was statistically significantly different compared with control mice (Fig. 3d).

Further pair-wise comparisons of the subgroups testing concentration-dependency of CD19^{+} BrdU^{+} B cells (not shown in Fig. 3d) did not show any trend when challenged with MI, OIT or BIT.

Cross-reactivity between methylisothiazolinone and octylisothiazolinone, and between methylisothiazolinone and benzisothiazolinone

Figure 4 presents some of the data shown in Figure 3a–d as line graphs of the means and SEM of the subgroups. Overall,

MI-sensitized mice showed the same immune response, whether being challenged with MI, OIT or BIT (Fig. 4). The differences in mean for the specific subgroups were accessed by the same preselected one-way ANOVA with post hoc Tukey's HSD test as described in Figure 3a–d. The same ear swelling was shown for MI, OIT and BIT, with no statistically significant difference in mean when comparing subgroups sensitized with the same concentration of MI (i.e. 0·13% MI, 0·4% MI or 1·2% MI) (Fig. 4a). Additionally, no statistical difference in CD4^{+} BrdU^{+} T cells, CD8^{+} BrdU^{+} T cells or CD19^{+} BrdU^{+} B cells was observed whether MI-sensitized mice were challenged with 0·4% MI, 0·7% OIT or 1·9% BIT, when comparing the preselected subgroups sensitized with the same concentration of MI (Fig. 4b–d).

Discussion

In this experimental study we investigated whether the immune response differed after a challenge phase with MI, BIT or OIT in MI-sensitized female CBA mice. MI induced significant concentration-dependent immune responses after a sensitization phase of three consecutive days. Notably, MI, OIT and BIT induced the same concentration-dependent inflammatory response with proliferation of CD4^{+} and CD8^{+}
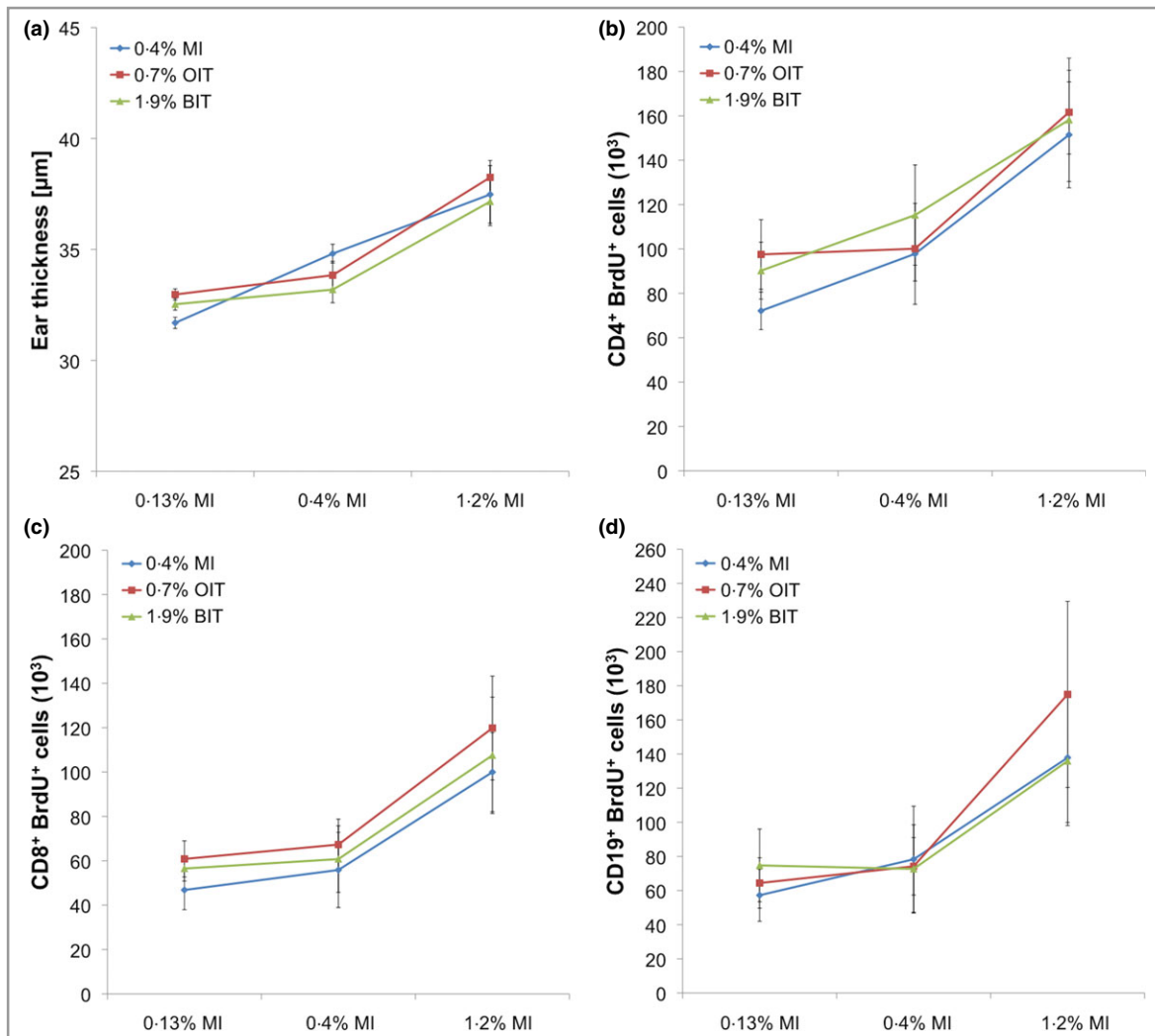
Fig 4. The mean and SEM (error bars) of (a) ear thickness, (b) CD4^{+} 5-bromo-2′-deoxyuridine (BrdU)[+] T cells, (c) CD8^{+} BrdU^{+} T cells and (d) CD19^{+} BrdU^{+} B cells in methylisothiazolinone (MI)-sensitized mice at three different concentrations (0·13% MI, 0·4% MI or 1·2% MI) and all dermally challenged with 0·4% MI, 0·7% octylisothiazolinone (OIT) or 1·9% benzisothiazolinone (BIT) at day 23. Eight mice in each group, based on two independent experiments. No statistically significant difference was shown for ear thickness, CD4^{+} BrdU^{+} cells, CD8^{+} BrdU^{+} cells or CD19^{+} BrdU^{+} cells whether the MI-sensitized mice were challenged with 0·4% MI, 0·7% OIT or 1·9% BIT, calculated by one-way ANOVA with (preselected) Tukey's post hoc test.

T cells and CD19^{+} B cells in MI-sensitized mice. This means that no significant difference was observed between the groups challenged with MI compared with the groups challenged with OIT or BIT. No such immune response was observed in the control mice.

It is noteworthy that cross-contamination was eliminated as purity analyses of each standard used showed that OIT and BIT were found in limited amounts in the purchased MI and vice versa. It was therefore not likely that the MI-sensitized mice were accidentally sensitized to OIT or BIT during the sensitization phase.

In accordance with our results, studies by Basketter *et al.* and Devos *et al.* found that MI has strong sensitizing capabilities.[18,23] In this study we used a verified modification of the local lymph node assay, where the sensitization phase lasted

for three consecutive days. Our data show that the mice were sensitized to MI, and, as expected, that 0·4% MI (the previously published EC3 value for MI) induced a threefold increase of cells in the draining lymph node (data not shown).

Thousands of European citizens are allegedly already sensitized to MI owing to daily skin contact with cosmetic products preserved with MI.[5–7] In December 2013, the Scientific Committee on Consumer Safety (SCCS), an independent advisory body of DG Santé (Directorate General, Consumer Safety and Health Protection), of the European Commission concluded that no safe level of MI could be determined for leave-on cosmetic products, and that for rinse-off cosmetic products only a maximum concentration of 15 ppm MI was safe from the point of view of sensitization.[24] Although the cosmetic

industry subsequently submitted cosmeto-vigilance data supporting the use of MI in concentrations up to 100 ppm in leave-on hair cosmetic products and rinse-off products to be safe for the consumer, the SCCS concluded in June 2015 (SCCS/1557/15) that the initial concern raised in the opinion SCCS/1521/13 remained.[25]

On top of the insufficient risk management of MI to date, the question of risk assessment of other isothiazolinones arises. In 2012, the SCCS concluded that BIT is not considered safe in respect to skin sensitization, as MI and MCI/MI clinically are important skin sensitizers. Our results show full cross-reactivity between MI and BIT, indicating that BIT is not safe for patients with MI contact allergy, if exposed to sufficiently high concentrations. In 1996, Geier and Schnuch concluded that no cross-reactivity existed between MCI/MI and BIT.[11] Similarly, recently published retrospective observational studies by Geier *et al.* and Aerts *et al.* could not confirm cross-reactivity between MI and BIT.[1,15] However, the degree of cross-reactivity will depend on the level of sensitization, that is, the concentration of MI at the time of sensitization. This will, in approximately two-thirds of all clinical cases, be up to 100 ppm MI, owing to its use as a preservative in cosmetic products for domestic use.[1,4–7] This should be compared with our experimentally chosen concentrations of MI (0·13%, 0·4% and 1·2%). Controlled murine studies, such as those presented herein, are, in general, superior in showing 'maximum scenarios' of cross-reactivity to the abovementioned observational studies when analysing cross-reactivity, as exposure is done under controlled circumstances and with known concentrations at the time of sensitization. Further, the selected and recommended patch test concentrations of BIT and OIT, used in many observational studies, may potentially be too low to detect cross-reactivity between MI, OIT and BIT in MI-sensitized individuals. Although, the patch test dose of MI has been optimized to 2000 ppm aq., the patch test doses and vehicles of BIT and OIT have not yet been optimized. Future clinical studies should therefore prioritize the detection of optimal patch test doses of BIT and OIT.

It is acknowledged that the extensive use of MI and other isothiazolinones in paints and other industrial chemical products massively expose workers, and to some extent consumers, to a risk of sensitization.[26,27] The verified use of BIT in paint and other industrial products may also be problematic owing to the hitherto shown cross-reactivity between MI and BIT.[26,27] However, the use of BIT may only be problematic to workers in direct skin contact with paints, that is, painters/decorators, as the evaporation of BIT from newly painted walls is negligible compared with MI.[28]

Octylisothiazolinone has not yet been assessed for use as preservative in cosmetic products in Europe. Theoretically, OIT may possess a lower sensitizing capability than MI, but as a potential risk of cross-reactivity between MI and OIT exists, it is of utmost importance to consider this in a future European risk assessment of OIT. Often, OIT is included in the more specialized patch test series, for example for painters, which is in accordance with OIT being an important allergen

that painters are exposed to.[13,27] Ten out of 20 targeted patch-tested Danish patients with OIT sensitization had relevant contact allergy, and 90% of the 10 had been exposed to OIT in an occupational setting, for example to paints.[29] The previously mentioned Belgian study by Aerts *et al.* indicated that cross-reactivity between MI and OIT may exist.[1] Approximately 40% of 15 Belgian patients allergic to MI with a positive patch test result to OIT had no relevant exposure to OIT, which was considered a sign of cross-reactivity between MI and OIT.[1]

Except for OIT, all EC3 values were chosen on the basis of published and verified EC3 values. However, no published EC3 value was found for OIT. We therefore tried to estimate the theoretical EC3 value for OIT, as, chemically, OIT is a homologue of MI.

The *in situ* chemical behaviour of MI, OIT and BIT in three-dimensional reconstructed human epidermis may be another approach to test cross-reactivity between MI, OIT and BIT, but such comprehensive studies have, to our knowledge, not yet been conducted. However, a French study recently concluded that cross-reactivity between MI and MCI did not exist owing to differences in the *in situ* chemical behaviour of MI and MCI.[30] It is important to emphasize that we found cross-reactivity between MI, OIT and BIT.

In conclusion, cross-reactivity was detected between MI and OIT, as well as between MI and BIT, when the potency of the chemical was taken into account in choice of challenge concentration. This new insight means that MI-sensitized individuals may react to other isothiazolinones such as OIT and BIT depending on exposure concentrations. Although the use of MI in European cosmetic products may be restricted according to the two recent opinions on MI (SCCS/1521/13 and SCCS/1557/15), consumers/workers with MI contact allergy may still experience problems with OIT and BIT in other consumer and/or occupational chemical products.

Acknowledgments

References

1 Aerts O, Baeck M, Constandt L *et al.* The dramatic increase in the rate of methylisothiazolinone contact allergy in Belgium: a multicentre study. *Contact Dermatitis* 2014; **71**:41–8.

2 Hosteing S, Meyer N, Waton J *et al.* Outbreak of contact sensitization to methylisothiazolinone: an analysis of French data from the REVIDAL-GERDA network. *Contact Dermatitis* 2014; **70**:262–9.

3 Lammintausta K, Aalto-Korte K, Ackerman L *et al.* An epidemic of contact allergy to methylisothiazolinone in Finland. *Contact Dermatitis* 2014; **70**:184–5.

4 Urwin R, Wilkinson M. Methylchloroisothiazolinone and methylisothiazolinone contact allergy: a new 'epidemic'. *Contact Dermatitis* 2013; **68**:253–5.

5 Gonçalo M, Goossens A. Whilst Rome burns: the epidemic of contact allergy to methylisothiazolinone. *Contact Dermatitis* 2013; **68**:257–8.

6 Anon. Incompetence and failure to regulate methylisothiazolinone. *Contact Dermatitis* 2015; **72**:353–4.

7 Schwensen JF, White IR, Thyssen JP *et al.* Failures in risk assessment and risk management for cosmetic preservatives in Europe and the impact on public health. *Contact Dermatitis* 2015; **73**:133–41.

8 Scientific Committee on Cosmetic Products and Non-food Products Intended for Consumers (SCCNFP). Opinion on methylisothiazolinone (P94). Available at: http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out270_en.pdf (last accessed 7 March 2016).

9 Scientific Committee on Consumer Safety. Opinion on benzisothiazolinone. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_099.pdf (last accessed 7 March 2016).

10 Bruze M, Gruvberger B, Persson K. Contact allergy to a contaminant in Kathon CG in the guinea pig. *Derm Beruf Umwelt* 1987; **35**:165–8.

11 Geier J, Schnuch A. No cross-reactivity between MCI/MI, benzisothiazolinone and octylisothiazolinone. *Contact Dermatitis* 1996; **34**:148–9.

12 Geier J, Lessmann H, Schnuch A, Uter W. Recent increase in allergic reactions to methylchloroisothiazolinone/methylisothiazolinone: is methylisothiazolinone the culprit? *Contact Dermatitis* 2012; **67**:334–41.

13 Mose AP, Lundov MD, Zachariae C *et al.* Occupational contact dermatitis in painters: an analysis of patch test data from the Danish Contact Dermatitis Group. *Contact Dermatitis* 2012; **67**:293–7.

14 Schwensen JF, Menné T, Andersen KE *et al.* Occupations at risk of developing contact allergy to isothiazolinones in Danish contact dermatitis patients: results from a Danish multicentre study (2009–2012). *Contact Dermatitis* 2014; **71**:295–302.

15 Geier J, Lessmann H, Schnuch A, Uter W. Concomitant reactivity to methylisothiazolinone, benzisothiazolinone, and octylisothiazolinone. International Network of Departments of Dermatology data, 2009–2013. *Contact Dermatitis* 2015; **72**:337–9.

16 Larsen JM, Geisler C, Nielsen MW *et al.* Cellular dynamics in the draining lymph nodes during sensitization and elicitation phases of contact hypersensitivity. *Contact Dermatitis* 2007; **57**:300–8.

17 Basketter DA, Smith Pease CK, Patlewicz GY. Contact allergy: the local lymph node assay for the prediction of hazard and risk. *Clin Exp Dermatol* 2003; **28**:218–21.

18 Basketter DA, Gilmour NJ, Wright ZM *et al.* Biocides: characterization of the allergenic hazard of methylisothiazolinone. *Cutan Ocul Toxicol* 2003; **22**:187–99.

19 Warbrick EV, Dearman RJ, Basketter DA, Kimber I. Influence of application vehicle on skin sensitization to methylchloroisothiazolinone/methylisothiazolinone: an analysis using the local lymph node assay. *Contact Dermatitis* 1999; **41**:325–9.

20 Roberts DW. Methylisothiazolinone is categorised as a strong sensitiser in the murine local lymph node assay. *Contact Dermatitis* 2013; **69**:261–2.

21 Gerberick GF, Ryan CA, Dearman RJ, Kimber I. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods* 2007; **41**:54–60.

22 Bollmann UE, Vollertsen J, Carmeliet J, Bester K. Dynamics of biocide emissions from buildings in a suburban stormwater catchment - concentrations, mass loads and emission processes. *Water Res* 2014; **56**:66–76.

23 Devos FC, Pollaris L, Van Den Broucke S *et al.* Methylisothiazolinone: dermal and respiratory immune responses in mice. *Toxicol Lett* 2015; **235**:179–88.

24 SCCS (Scientific Committee on Consumer Safety). Opinion on methylisothiazolinone (P94). Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_145.pdf (last accessed 7 March 2016).

25 SCCS (Scientific Committee on Consumer Safety). Opinion on methylisothiazolinone (MI) (P94) (sensitization only). Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_178.pdf (last accessed 7 March 2016).

26 Schwensen JF, Lundov MD, Bossi R *et al.* Methylisothiazolinone and benzisothiazolinone are widely used in paint: a multicentre study of paints from five European countries. *Contact Dermatitis* 2015; **72**:127–38.

27 Friis UF, Menné T, Flyvholm MA *et al.* Isothiazolinones in commercial products at Danish workplaces. *Contact Dermatitis* 2014; **71**:65–74.

28 Lundov MD, Kolarik B, Bossi R *et al.* Emission of isothiazolinones from water-based paints. *Environ Sci Technol* 2014; **48**:6989–94.

29 Mose AP, Frost S, Ohlund U, Andersen KE. Allergic contact dermatitis from octylisothiazolinone. *Contact Dermatitis* 2013; **69**:49–52.

30 Debeuckelaere C, Moussallieh FM, Elbayed K *et al. In situ* chemical behaviour of methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI) in reconstructed human epidermis: a new approach to the cross-reactivity issue. *Contact Dermatitis* 2016; **74**:159–67.

4.4 The epidemic of methylisothiazolinone: a European prospective study – Manuscript IV

- 6.0% (205/3434) of consecutive patch-tested patients in eight European countries (11 clinics) had MI contact allergy.
- The dermatitis most often affected hands (43.4%), face (32.7%), arms (14.6%), eyelids (11.7%), neck (10.2%), legs (10.2%), ano-genital area (4.9%), feet (2.9%) and scalp (1.5%).
- Widespread dermatitis (defined as dermatitis at more than 3 anatomical sites) was found in 12.7% of patients with MI contact allergy.
- Relevant contact allergy to MI was found in 72.7% (149/205) of the patients with MI contact allergy.
- 88.6% (132/149) were exposed to products containing MI while 11.4% (17/149) were exposed to products containing MCI/MI.
- In most cases, relevant MI contact allergy was due to exposure to cosmetic products (83.2%; 124/149) in the individuals' domestic and/or occupational environment.
- 19.5% were exposed to leave-on and rinse-off cosmetic products.
- 24.8% were exposed only to leave-on cosmetic products.
- 38.9% were exposed only to rinse-off cosmetic products.
- Occupational contact dermatitis due to MI contact allergy was seen in 16.8% (n=25) of patients with relevant contact allergy to MI. This was mainly due to cleaning agents, water-based paints and lacquers, or cosmetic and household products at the workplace.
- In 7.3% (n=15) of the cases, being in a newly painted room had resulted in allergic reactions.

The epidemic of methylisothiazolinone: a European prospective study

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Summary

Background. The use of methylisothiazolinone (MI) in cosmetic products has caused an unprecedented epidemic of MI contact allergy. Current data concerning exposures at a European level are required.

Objectives. To describe demographics and MI exposures for European patients with MI contact allergy.

Methods. Eleven European dermatology departments from eight European countries prospectively collected data between 1 May and 31 October 2015 among consecutive patients who had positive patch test reactions to MI (2000 ppm aq.).

Results. A total of 6.0% (205/3434; range 2.6–13.0%) of patients had positive patch test reactions to MI. Dermatitis most frequently affected the hands (43.4%), face (32.7%), arms (14.6%), and eyelids (11.7%); 12.7% had widespread dermatitis. For 72.7% (149/205), MI contact allergy was currently relevant mainly because of exposure to cosmetic products (83.2%; 124/149). Of these 124 patients, 19.5% were exposed to leave-on and rinse-off cosmetic products, 24.8% only to leave-on cosmetic products and 38.9% only to rinse-off cosmetic products containing MI or methylchloroisothiazolinone/MI. The majority of these (79%) noted onset of their dermatitis between 2013 and 2015. Fifteen patients (7.3%) had previously experienced allergic reactions when they were in newly painted rooms.

Conclusion. Clinically relevant MI contact allergy remains prevalent across European countries, mainly because of exposure to rinse-off and leave-on cosmetic products.

Key words: allergic contact dermatitis; CAS no. 2682-20-4; cosmetics; exposure; methylchloroisothiazolinone; methylisothiazolinone.

Contact Dermatitis

1

Methylisothiazolinone (MI; CAS no. 2682-20-4) was already shown to be a sensitizer in humans and guinea-pigs in the mid-1980s (1, 2). The first occupational cases of allergic contact dermatitis caused by MI were reported in 2004, and the first report on MI contact allergy related to cosmetic products was published in 2010 (3, 4). Since then, the continued use of MI as a preservative in cosmetic, household and industrial chemical products has resulted in an unprecedented increase in the prevalence of contact allergy to MI in Europe (5–13). Although MI-containing cosmetic products and household products account for most cases of contact allergy to MI, its use in products for occupational use, for example water-based paints and metalworking fluids, has also caused skin problems in workers (5, 13–18).

Scientists and national health or environmental authorities tried for several years to raise awareness of the European outbreak of contact allergy to MI (12, 19, 20). In 2013, the European Commission (EC) requested an opinion (SCCS/1521/13) from the Scientific Committee of Consumer Safety (SCCS) (21). The SCCS concluded that the European consumer was not sufficiently protected with regard to sensitization with a concentration of 100 ppm MI in cosmetic products (21): 'For leave-on cosmetic products (including "wet wipes"), no safe concentrations of MI for induction of contact allergy or elicitation have been adequately demonstrated. For rinse-off cosmetic products, a concentration of 15 ppm (0.0015%) MI is considered safe for the consumer from the view of induction of contact allergy. However, no information is available on elicitation.' Subsequent to this opinion, the European cosmetics industry submitted an additional dossier of data to support the safe use of MI at 100 ppm in rinse-off cosmetic products and leave-on hair care products (22). The SCCS, however, arrived at the same conclusion in the SCCS/1557/15 opinion as in its earlier SCCS/152/13 opinion (21, 22). In spring 2016, EU member states agreed on a ban on the use of MI in leave-on cosmetic products, which will be effective from 1 January 2017 after a 6-month transition period, during which the cosmetic industry may still produce leave-on cosmetic products containing MI.

The EC held a public consultation on the use of MI in rinse-off cosmetic products from 1 April 2016 to 1 July 2016, to seek the opinions of interested parties (23). Here, the EC proposed accepting the advice given in opinion SCCS/1557/15 to restrict the use of MI in rinse-off cosmetic products to 15 ppm MI (22, 23). Although this is an important step, the European consumer will continue to be exposed to MI at up to 100 ppm in rinse-off cosmetic products until the proposal has been implemented. It is therefore important to continuously monitor the trend of

MI contact allergy in the European population. Hitherto, no prospective European multicentre study has been performed. The purpose of this study was to investigate patients with MI contact allergy and their exposures to cosmetic products, household products and industrial chemical products containing MI during a defined period.

Materials and Methods

This prospective multicentre study was conducted at 11 centres in eight European countries (for the case record form, see Table S1; for technical details, see Table 1). During the study period from 1 May 2015 to 30 October 2015, patients with positive patch test reactions (reactions designated as +, ++, or +++) to 2000 ppm (0.2%) MI aq. were included.

Patch testing was performed according to ESCD recommendations (24). The patch tests were applied to the upper back and occluded for 2 days. In most of the centres, readings were performed on day (D) 2, D3/D4, and D7. All patients were patch tested with the European baseline series. All centres used their usual routines, and different patch test systems (Table 1) were therefore used. Demographics, patch test results and exposures to MI and methylchloroisothiazolinone (MCI)/MI were recorded for all MI patch test-positive patients in each clinic. The patients with positive MI patch test results were asked to bring all their cosmetic products, toiletries, cleaning products and products for occupational use that they used in their domestic and occupational environments. All products preserved with MI or MCI/MI were recorded with regard to product type and use (domestic versus occupational). If the substance was relevant for the present contact dermatitis, additional information, such as manufacturer and specific product name, was registered.

Descriptive analyses of the anonymized data were performed at the National Allergy Research Centre, according to pertinent guidelines (25), with SPSS™ (SPSS™ Statistics, Chicago, IL, USA; IBM PASW Statistics) for Windows™, edition 20.0.

Results

A total of 205 patients had positive patch test reactions to MI (2000 ppm aq.) among 3434 consecutively patch tested patients (6.0%, 95% confidence interval: 5.2–6.8%; range 2.6–13.0%) (Table 1). Table 2 shows that females were predominant in the group (69.8%; 143/205) of patients with MI contact allergy; 23.4% (48/205) had previous or current atopic dermatitis (not shown in Table 2), and the mean age was 47.0 years. The dermatitis was primarily localized on the hands and the facial region. One in every 10 had widespread dermatitis,

2

Table 1. Overview of the data of 205 European patients with methylisothiazolinone (MI) contact allergy prospectively collected in 11 dermatology departments in eight European countries

Country	Centre	Contact allergy to MI, % (n/n$_{total}$)	Test system and producer of MI patch test material
Belgium	Leuven	7.3 (22/302)	IQ Ultra™, Chemotechnique Diagnostics
Denmark	Bispebjerg	5.0 (12/241)	Finn Chambers®, Chemotechnique Diagnostics
	Gentofte	5.2 (27/519)	Finn Chambers®, Chemotechnique Diagnostics
	Odense	5.8 (15/257)	Finn Chambers®, Chemotechnique Diagnostics
Finland	Finnish Institute of Occupational Health	13.0 (7/54)	Finn Chambers®, Chemotechnique Diagnostics
United Kingdom	Leeds	5.2 (21/404)	IQ Ultra™, Chemotechnique Diagnostics
	London	5.1 (27/526)	Finn Chambers®, Chemotechnique Diagnostics
Italy	Bari	2.6 (8/313)	Al test®, Euromedical, Calolziocorte, LC, Italy
Portugal	Coimbra	8.5 (15/177)	IQ Ultra™, Chemotechnique Diagnostics
Spain	Barcelona	6.7 (17/255)	Finn Chambers®, Chemotechnique Diagnostics
Sweden	Malmö	8.8 (34/386)	Finn Chambers®, Chemotechnique Diagnostics

Table 2. Demographics for 205 European patients with methylisothiazolinone contact allergy prospectively collected in 11 dermatology departments in eight European countries

Age (years), mean (n$_{total}$)	47.0 (201)
1–30, % (n)	21.4 (43)
31–50, % (n)	32.2 (65)
>50, % (n)	46.3 (93)
Female sex, % (n/n$_{total}$)	69.8 (143/205)
Previous atopic dermatitis, % (n/n$_{total}$)	7.3 (15/205)
Current atopic dermatitis, % (n/n$_{total}$)	18.0 (37/205)
No present contact dermatitis, % (n/n$_{total}$)	8.3 (17/205)
Anatomical site of contact dermatitis, % (n/n$_{total}$)	
Widespread	12.7 (26/205)
Hands	43.4 (89/205)
Face	32.7 (67/205)
Arms	14.6 (30/205)
Trunk	13.7 (28/205)
Eyelids	11.7 (24/205)
Neck	10.2 (21/205)
Legs	10.2 (21/205)
Anogenital	4.9 (10/205)
Feet	2.9 (6/205)
Scalp	1.5 (3/205)

here defined as dermatitis involvement of more than three anatomical sites (Table 2). No notable difference in the location of dermatitis was observed when MI contact allergy was stratified for current or past relevance.

Patch test results with selected allergens from the European baseline series for the 205 patients with MI contact allergy are shown in Table 3. MCI/MI elicited positive patch test reactions in 64.2% (129/201) of MI-positive patients. A total of 15% of the patients (n = 32) previously had a positive patch test reaction to either MI or MCI/MI. Polysensitization, defined as the presence of contact allergy to three or more unrelated allergens, was registered in 24.9% (n = 51) of MI-positive patients.

A total of 72.7% (149/205) MI-positive patients had current and certain relevance of their MI contact allergy resulting from exposures primarily to rinse-off and leave-on cosmetic products containing MI or MCI/MI (Table 4). A total of 83.2% (124/149) were exposed to cosmetic products containing MI or MCI/MI in their domestic and/or occupational environment: 58.4% (n = 87) were exposed to rinse-off cosmetic products, in comparison with 44.3% (n = 66) exposed to leave-on cosmetic products. Twenty-nine patients with relevant MI contact allergy were, however, exposed to rinse-off and leave-on cosmetic products containing MI or MCI/MI. Of the 149 patients with relevant MI contact allergy, 99 were exposed only to cosmetic products, 19 were exposed only to household products (primarily dishwashing detergent), 23 were exposed to cosmetic products and household products, 4 were exposed only to water-based paint and lacquer, 1 was exposed to water-based paint and cosmetic products, 1 was exposed to water-based paint, cosmetic products, and household products, and 2 were exposed only to chemical products for occupational use (glue and surface treatment for floors). Office work, healthcare work and cleaning were the most common occupations registered for the 205 patients with MI contact allergy (Table S2).

Among the 149 patients with relevant MI contact allergy who were exposed to products containing MI or MCI/MI, more than half of all patients with relevant MI contact allergy were exposed to more than one product containing MI or MCI/MI: 71 (47.7%) were exposed to one product containing MI or MCI/MI, 32 (21.5%) were exposed to two products, 20 (13.4%) were exposed to three products, 12 (8.1%) were exposed to four products, and 14 (9.4%) were exposed to more than four products. Almost 90% of patients with relevant MI contact allergy (88.6%; 132/149) were exposed to products

Table 3. Patch test results for 205 European patients with methylisothiazolinone contact allergy, and results of additional patch testing with selected allergens from the European baseline series

Allergen (%, n/n$_{total}$)	Patch test reaction				
	Weak positive reaction (1+)	Strong positive reaction (2+)	Extreme positive reaction (3+)	Negative	Doubtful
Methylisothiazolinone	25.4 (52/205)	56.6 (116/205)	18.0 (37/205)	0.0 (0/205)	0.0 (0/205)
Methylchloroisothiazolinone/ methylisothiazolinone	29.4 (59/201)	23.9 (48/201)	10.9 (22/201)	31.3 (63/201)	4.5 (9/201)
Other selected allergens from the European baseline series					
Fragrance mix I	5.9 (11/185)	9.7 (18/185)	1.1 (2/185)	82.7 (153/185)	0.5 (1/185)
Fragrance mix II	6.6 (12/183)	4.4 (8/183)	3.8 (7/183)	84.7 (155/183)	0.5 (1/183)
Myroxylon pereirae	5.5 (10/183)	0.5 (1/183)	0.5 (1/183)	92.9 (170/183)	0.5 (1/183)
Formaldehyde	2.7 (5/184)	3.8 (7/184)	0.5 (1/184)	91.3 (168/184)	1.6 (3/184)
Quaternium-15	0.5 (1/182)	1.6 (3/182)	0.0 (0/182)	97.8 (178/182)	0.0 (0/182)
Paraben mix	0.0 (0/181)	0.6 (1/181)	0.0 (0/181)	99.4 (180/181)	0.0 (0/181)
Nickel sulfate	4.3 (8/186)	14.5 (27/186)	3.8 (7/186)	76.3 (142/186)	0.0 (0/186)
Potassium dichromate	1.6 (3/182)	1.6 (3/182)	0.0 (0/182)	95.6 (174/182)	0.5 (1/182)
Cobalt chloride	3.9 (7/181)	2.2 (4/181)	1.7 (3/181)	91.7 (166/181)	0.6 (1/181)

Table 4. Exposures to products containing methylisothiazolinone (MI) or methylchloroisothiazolinone/MI in 149 patients with clinically relevant MI contact allergy

Product category	% (n)
Both leave-on and rinse-off cosmetic products	19.5 (29)
Rinse-off cosmetic products	38.9 (58)
Leave-on cosmetic products (including wet wipes)	24.8 (37)
Household products without exposure to cosmetic products*	12.8 (19)
Paints or chemical products for occupational use	4.0 (6)
Total	*100 (149)*

Of the patients, 83.2% (n = 124) were exposed to cosmetic products. *A total of 42 patients were exposed to household products: 19 only to household products, and 23 to both household products and primarily cosmetic products.

containing MI (the rest were exposed to products containing MCI/MI): 91.7% (n = 121) of these were exposed to domestic products, 18.2% (n = 24) were exposed to chemical products for occupational use or cosmetic products at their workplace, and 10.7% (n = 13) were exposed in both their domestic and occupational environments. Patients with relevant MI contact allergy had less exposure to products containing MCI/MI (36.9%; n = 55) in terms of domestic exposures (94.5%; n = 52) and occupational exposures (18.2%; n = 10). Only 12.7% (n = 7) were exposed to products containing MCI/MI in their domestic and occupational environments.

A total of 16.8% (n = 25) of patients with relevant MI contact allergy had occupational contact dermatitis resulting from occupational exposure to products containing MI or MCI/MI: cleaning agents (n = 6), water-based paint (n = 4), glue (n = 1), lacquer (n = 1),

and/or cosmetic products and household products (n = 13). Occupational contact dermatitis was seen most frequently in: nurses (n = 4), hairdressers (n = 4), cleaners (n = 3), cosmetologists (n = 2), factory workers (e.g. in glue production) (n = 2), painters (n = 2), carer (n = 1), machinist (n = 1), and others (n = 7).

The year of onset of dermatitis in the 149 patients with relevant MI contact allergy is shown in Fig. 1. Approximately 79% (100/126) developed contact dermatitis between 2013 and 2015 (until 30 October 2015).

Table 5 shows the patients' exposures to MI-containing products. Rinse-off cosmetic products containing MI were frequently registered: shampoos, baths/shower gels, and
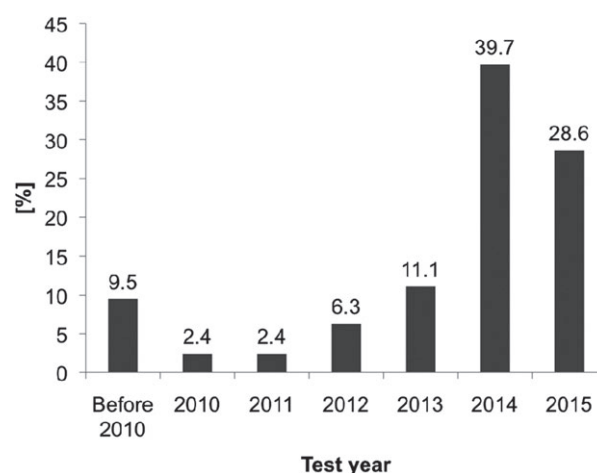
Fig. 1. The year of onset of contact dermatitis in 149 patients with clinically relevant contact allergy to methylisothiazolinone. n$_{total}$ = 126 (missing data: 23).

Table 5. Exposures to domestic and occupational products containing methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI)/MI in 205 European patients with MI contact allergy

Product category	MI, % (n)	MCI/MI, % (n)
Shampoo	15.7 (45)	22.5 (25)
Dishwashing liquid	12.6 (36)	8.1 (9)
Face cream/lotion	8.0 (23)	3.6 (4)
Baths/shower gel	8.0 (23)	14.4 (16)
Body cream/lotion	7.0 (20)	5.4 (6)
Liquid soap	6.6 (19)	6.3 (7)
Cleansing agent	5.9 (17)	11.7 (13)
Make-up remover	4.5 (13)	1.8 (2)
Conditioner	3.5 (10)	6.3 (7)
Wet wipes	3.1 (9)	5.4 (6)
Cream/lotion (unspecified)	2.8 (8)	0.0 (0)
Hairstyling (gel/mousse)	2.4 (7)	1.8 (2)
Paint	2.4 (7)	1.8 (2)
Household cleansing spray	1.7 (5)	1.8 (2)
Eye cream	1.4 (4)	1.8 (2)
Sunscreen/self-tanning	1.4 (4)	0.0 (0)
Hand cream/lotion	1.0 (3)	0.0 (0)
Face mask	1.0 (3)	0.0 (0)
Cream/lotion for feet	0.7 (2)	0.0 (0)
Deodorant (unspecified)	0.7 (2)	0.0 (0)
Glues	0.7 (2)	0.0 (0)
Hairstyling product (unspecified)	0.7 (2)	2.7 (3)
Deodorant (roll-on/stick)	0.3 (1)	0.0 (0)
Deodorant (spray)	0.3 (1)	0.9 (1)
Hairstyling spray	0.3 (1)	0.0 (0)
Make-up (unspecified)	0.3 (1)	0.0 (0)
Rinse-off (unspecified)	0.3 (1)	0.0 (0)
Shaving product	0.3 (1)	0.0 (0)
Others	5.6 (16)	3.6 (4)
Total	*100 (286)*	*100 (111)*

Of the patients, 73.1% (n = 144) and 32.8% (n = 63) were exposed to MI and MCI/MI, respectively. Patients may have been exposed to more than one product.

liquid soaps. However, leave-on cosmetic products, such as creams/lotions and body creams/lotions were also frequently registered (Table 5). Some brands of product were mentioned more frequently than others as having caused allergic contact dermatitis in 149 patients with relevant MI contact allergy: Clarins® (n = 20), Pantene™ (Procter & Gamble) (n = 16), TRESemmé® (Unilever) (n = 13), Nivea® (Beiersdorf AG) (n = 13), Fairy® (Procter & Gamble) (n = 10), Head & Shoulders® (Procter & Gamble) (n = 8), Dove® (Unilever) (n = 5), Dreft® (Procter & Gamble) (n = 4), L'Oréal (n = 4), and Revlon® (n = 4).

A total of 7.3% (15/205) MI-positive patients had previously experienced allergic reactions when they were in newly painted rooms: contact dermatitis (n = 13), rhinitis (n = 2), and/or conjunctivitis (n = 1). However, none experienced asthma. Eight patients (3.9%) experienced allergic reactions to airborne exposures other than paint, mainly cleaning agents.

Discussion

This multicentre study of 205 patients with MI contact allergy from eight European countries showed that MI contact allergy remains frequent across all countries; however, there are national differences. Dermatitis in patients with MI contact allergy was most often localized to the hands and face, and 72.7% of the patients had current relevance of their MI contact allergy, primarily resulting from the use of rinse-off and leave-on cosmetic products containing MI or MCI/MI.

To our knowledge, this is the first prospective study to investigate the prevalence of MI contact allergy and to perform exposure analysis in a broad selection of European patients with MI contact allergy. Our results are in line with those of prior retrospective observational studies of consecutive patients with MI contact allergy (5–13, 26). The majority of patients with relevant MI contact allergy (79%; 100/126) had an onset of their contact dermatitis between 2013 and 2015. However, the number of patients with MI contact allergy in 2015 is probably underestimated, as we only included patients until October 2015.

Notably, our results show that 83.4% of patients with relevant MI contact allergy had been exposed to cosmetic products containing MI or MCI/MI. The epidemic of MI contact allergy has been driven by the use of MI in cosmetic products. The use of MI in rinse-off cosmetic products is of particular concern, as its use at the currently permitted concentration up to a maximum of 100 ppm can elicit contact dermatitis in patients with MI contact allergy, according to use tests (27). The use of MI in rinse-off cosmetic products may, purely on the basis of our exposure results, be of even more concern than the use of MI in leave-on cosmetic products, as our patients were exposed to many more rinse-off cosmetic products than leave-on cosmetic products. Exposure analyses on the use of MI in cosmetic products based on the European mandatory ingredient labelling have previously provided estimates of exposure varying between ~0.5% and 3.3% (28–30). In one analysis, it was shown that the frequency of the use of MI in leave-on and rinse-off cosmetic products was approximately equal (28). According to our data, the restriction on the use of MI in rinse-off cosmetic products to 15 ppm MI, as previously suggested by the SCCS (22), seems to be justified. Currently, a public hearing held by the EC is being conducted concerning its implementation (23).

Water-based paint is a source of clinically relevant exposure to MI (17, 31–33). It has recently been

recognized that MI is extensively used as a preservative in European water-based paints, although in varying concentrations (0.7–180.9 ppm) (17). MI evaporates from newly painted surfaces, and may result in airborne allergic contact dermatitis (15). Our data show that 16.8% of the patients with relevant MI contact allergy were exposed to products for occupational use (including water-based paints), cosmetic products and household products containing MI or MCI/MI at their workplace. This is in accordance with the findings of other European studies (5, 13). Until March 2016, MI was not officially classified as a skin sensitizer in the EU. Accordingly, industry could legally omit information regarding the content of MI in their chemical products for occupational use, as long as the rules of self-classification according to the regulation on classification, labelling and packaging of substances and mixtures (CLP Regulation) were adhered to (34). This has previously been shown to be the case for water-based paints purchased in Europe (17). However, the Committee for Risk Assessment concluded, on 11 March 2016, that MI should be classified as 'Skin Sens 1A, H317', with a specific concentration limit of 0.0015% (35). We suggest that the use of MI should be fully restricted in water-based paint in order to protect European workers and consumers, as water-based paints can be preserved without the use of MI (17).

Interestingly, we found a relatively high frequency of polysensitization and simultaneous contact allergy to fragrance mix I, fragrance II and formaldehyde in patients with MI contact allergy, which is in accordance with a recent Spanish and Swedish study (Table 3) (36, 37). It has previously been estimated that the frequencies of contact allergy to fragrance mix I, fragrance II and formaldehyde are approximately doubled in patients with MI contact allergy as compared with the frequency in patients without MI contact allergy (37, 38). However, our results on polysensitization should be interpreted with caution, as it is possible that not all positive patch test results were registered (Table S1).

Nearly 65% of all patients with MI contact allergy had positive patch test reactions to MCI/MI, which is similar to what has been found in other studies (7, 28, 38). Hitherto, immunological cross-reactivity between isothiazolinones

has mainly been discussed in observational studies (5, 39–41). A recent French experimental study of three-dimensionally reconstructed human epidermis concluded that immunological cross-reactivity between MI and MCI was unlikely, as their *in situ* chemical behaviour was different (43). However, a recent murine study based on a modified local lymph node assay concluded that immunological cross-reactivity existed between MI, octylisothiazolinone, and benzisothiazolinone (44). Our current study does not include data that may or may not verify potential immunological cross-reactivity between isothiazolinones.

In conclusion, it is of concern that clinically relevant MI contact allergy remains prevalent across European countries. MI used in rinse-off and leave-on cosmetic products continues to cause problems for the European consumer. Cosmetics producers have not managed to self-regulate the use of MI in their products on the European market, and the use of MI in a number of cosmetic brands is of particular concern, as these cosmetic brands were frequently recorded in our study.

The planned European restriction on the use of MI in cosmetic products and adherence to the CLP Regulation are important and necessary to ensure the population's health.

Acknowledgements

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Pre-printed paper form for registration of demographics, patch test results and exposures to methylisothiazolinone (MI) and methylchloroisothiazolinone in combination with MI (MCI/MI) for each patient with MI contact allergy included in the prospective study conducted at 11 centres in eight European countries from 1 May 2015 to 30 October 2015.
Table S2. Occupational classification of 205 European patients with methylisothiazolinone contact allergy.

References

1 Bruze M, Fregert S, Gruvberger B, Persson K. Contact allergy to the active ingredients of Kathon CG in the guinea pig. *Acta Derm Venereol* 1987: **67**: 315–320.
2 Bruze M, Dahlquist I, Fregert S et al. Contact allergy to the active ingredients of Kathon CG. *Contact Dermatitis* 1987: **16**: 183–188.
3 Isaksson M, Gruvberger B, Bruze M. Occupational contact allergy and dermatitis from methylisothiazolinone after contact with wallcovering glue and after a chemical burn from a biocide. *Dermatitis* 2004: **15**: 201–205.
4 García-Gavín J, Vansina S, Kerre S et al. Methylisothiazolinone, an emerging allergen in cosmetics? *Contact Dermatitis* 2010: **63**: 96–101.

Contact Dermatitis

5 Aerts O, Baeck M, Constandt L et al. The dramatic increase in the rate of methylisothiazolinone contact allergy in Belgium: a multicentre study. *Contact Dermatitis* 2014: **71**: 41–48.

6 Gameiro A, Coutinho I, Ramos L, Gonçalo M. Methylisothiazolinone: second 'epidemic' of isothiazolinone sensitization. *Contact Dermatitis* 2014: **70**: 242–243.

7 Geier J, Lessmann H, Schnuch A, Uter W. Recent increase in allergic reactions to methylchloroisothiazoli-none/methylisothiazolinone: is methylisothiazolinone the culprit? *Contact Dermatitis* 2012: **67**: 334–341.

8 Hosteing S, Meyer N, Waton J et al. Outbreak of contact sensitization to methylisothiazolinone: an analysis of French data from the REVIDAL-GERDA network. *Contact Dermatitis* 2014: **70**: 262–269.

9 Johnston G A, Contributing members of the British Society for Cutaneous Allergy (BSCA). The rise in prevalence of contact allergy to methylisothiazolinone in the British Isles. *Contact Dermatitis* 2014: **70**: 238–240.

10 Lammintausta K, Aalto-Korte K, Ackerman L et al. An epidemic of contact allergy to methylisothiazolinone in Finland. *Contact Dermatitis* 2014: **70**: 184–185.

11 Madsen J T, Andersen K E. Further evidence of the methylisothiazolinone epidemic. *Contact Dermatitis* 2014: **70**: 246–247.

12 Schwensen J F, White I R, Thyssen J P et al. Failures in risk assessment and risk management for cosmetic preservatives in Europe and the impact on public health. *Contact Dermatitis* 2015: **75**: 133–141.

13 Uter W, Geier J, Bauer A, Schnuch A. Risk factors associated with methylisothiazolinone contact sensitization. *Contact Dermatitis* 2013: **69**: 334–341.

14 Friis U F, Menné T, Flyvholm M A et al. Isothiazolinones in commercial products at Danish workplaces. *Contact Dermatitis* 2014: **71**: 65–74.

15 Lundov M D, Kolarik B, Bossi R et al. Emission of isothiazolinones from water-based paints. *Environ Sci Technol* 2014: **48**: 6989–6994.

16 Mose A P, Lundov M D, Zachariae C et al. Occupational contact dermatitis in painters: an analysis of patch test data from the Danish Contact Dermatitis Group. *Contact Dermatitis* 2012: **69**: 49–52.

17 Schwensen J F, Lundov M D, Bossi R et al. Methylisothiazolinone and benzisothiazolinone are widely used in paint: a multicentre study of paints from five European countries. *Contact Dermatitis* 2015: **72**: 127–138.

18 Urwin R, Warburton K, Carder M et al. Methylchloroisothiazolinone and methylisothiazolinone contact allergy: an occupational perspective. *Contact Dermatitis* 2015: **72**: 381–386.

19 Gonçalo M, Goossens A. While Rome burns: the epidemic of contact allergy to methylisothiazolinone. *Contact Dermatitis* 2013: **68**: 257–258.

20 Bruze M, Uter W, Gonçalo M et al. Incompetence and failure to regulate methylisothiazolinone. *Contact Dermatitis* 2015: **72**: 353–354.

21 Scientific Committee on Consumer Safety (SCCS). Opinion on methylisothiazolinone (P94) – Submission II (sensitisation only). SCCS/1521/13, 2014 March 27. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_145.pdf (last accessed 11 October 2016).

22 Scientific Committee on Consumer Safety (SCCS). Opinion on methylisothiazolinone (P94) – Submission III. SCCS/1557/15, 2015 December 15. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_178.pdf (last accessed 11 October 2016).

23 European Commission (EC). Directorate General for Internal Market, Industry, Entrepreneurship and SMEs. Public consultation on Methylisothiazolinone (MI) in the framework of Regulation (EC) No. 1223/209 – rinse-off cosmetic products, 01/04/2016. Available at: http://ec.europa.eu/growth/tools-databases/newsroom/cf/itemdetail.cfm?item_id=8740&lang=en&title=Public-consultation-on-Methylisothiazolinone-8MI9-in-the-framework-of-Regulation-EC9-NoE-1223F2009--rinse-off-cosmetic-products (last accessed 11 October 2016).

24 Johansen J D, Aalto-Korte K, Agner T et al. European Society of Contact Dermatitis guideline for diagnostic patch testing – recommendations on best practice. *Contact Dermatitis* 2015: **73**: 195–221.

25 Uter W, Schnuch A, Gefeller O. Guidelines for the descriptive presentation and statistical analysis of contact allergy data. *Contact Dermatitis* 2004: **51**: 47–56.

26 Wilkinson K L, Wilkinson M. Contact allergy to methylisothiazolinone: has there been any change? Experience of a UK centre. *Contact Dermatitis* 2015: **72**: 398–400.

27 Yazar K, Lundov M D, Faurschou A et al. Methylisothiazolinone in rinse-off products causes allergic contact dermatitis: a repeated open-application study. *Br J Dermatol* 2015: **173**: 115–122.

28 Lundov M D, Opstrup M S, Johansen J D. Methylisothiazolinone contact allergy – growing epidemic. *Contact Dermatitis* 2013: **69**: 271–275.

29 Yazar K, Johnsson S, Lind M L et al. Preservatives and fragrances in selected consumer-available cosmetics and detergents. *Contact Dermatitis* 2011: **64**: 265–272.

30 Uter W, Yazar K, Kratz E M et al. Coupled exposure to ingredients of cosmetic products: II. Preservatives. *Contact Dermatitis* 2014: **70**: 219–226.

31 Alwan W, White I R, Banerjee P. Presumed airborne contact allergy to methylisothiazolinone causing severe facial dermatitis and respiratory difficulty. *Contact Dermatitis* 2014: **70**: 320–321.

32 Kaae J, Menné T, Thyssen J P. Presumed primary contact sensitization to methylisothiazolinone from paint: a chemical that became airborne. *Contact Dermatitis* 2012: **66**: 341–342.

33 Lundov M D, Friis U F, Menné T, Johansen J D. Methylisothiazolinone in paint forces a patient out of her apartment. *Contact Dermatitis* 2013: **69**: 252–253.

34 European Chemicals Agency (ECHA). Guidance on labelling and packaging in accordance with regulation (EC) No. 1272/2008, Reference ECHA-11-G-04-EN 2011 April. Available at: https://echa.europa.eu/documents/10162/13562/clp_labelling_en.pdf (last accessed 11 October 2016).

35 European Chemicals Agency (ECHA). Committee for risk assessment, RAC. Opinion, proposing harmonised classification and labelling at EU level of 2-methylisothiazol-3(2H)-one (ISO), Adopted 10 March 2016. Available at: http://echa.europa.eu/documents/10162/b32e77aa-b720-4f66-ad16-a413dadf1631 (last accessed 11 October 2016).

36 Martin-Gorgojo A, Curto-Barredo L, Rovira-López R et al. Is methylisothiazolinone contact allergy a risk factor for polysensitization? *Contact Dermatitis* 2015: **72**: 400–402.

37 Pontén A, Bruze M, Engfeldt M et al. Concomitant contact allergies to formaldehyde, methylchloroisothiazoli-none/methylisothiazolinone, methylisothiazolinone, and fragrance mixes I and II. *Contact Dermatitis* 2016: **75**: 285–289.

38 Pontén A, Aalto-Korte K, Agner T et al. Patch testing with 2.0% (0.60 mg/cm^2) formaldehyde instead of 1.0% (0.30 mg/cm^2) detects significantly more contact allergy. *Contact Dermatitis* 2013: **68**: 50–53.

39 Geier J, Schnuch A. No cross-reactivity between MCI/MI, benzisothiazolinone and octylisothiazolinone. *Contact Dermatitis* 1996: **34**: 148–149.

40 Geier J, Lessmann H, Schnuch A, Uter W. Concomitant reactivity to methylisothiazolinone, benzisothiazoinone, and octylisothiazolinone. International Network of Departments of Dermatology data, 2009–2013. *Contact Dermatitis* 2015: **72**: 337–339.

41 Isaksson M, Bruze M, Gruvberger B. Cross-reactivity between methylchloroisothiazoli-none/methylisothiazolinone, methylisothiazolinone, and other isothiazolinones in workers at a plant producing binders for paints and glues. *Contact Dermatitis* 2008: **58**: 60–62.

42 Isaksson M, Gruvberger B, Bruze M. Patch testing with serial dilutions of various isothiazolinones in patients hypersensitive to methylchloroisothiazoli-none/methylisothiazolinone. *Contact Dermatitis* 2014: **70**: 270–275.

43 Debeuckelaere C, Moussalieh F M, Elbayed K et al. In situ chemical behaviour of methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI) in reconstructed human epidermis: a new approach to the cross-reactivity issue. *Contact Dermatitis* 2016: **74**: 159–167.

44 Schwensen J F, Menné Bonefeld C, Zachariae C et al. Cross-reactivity between methylisothiazolinone, octylisothiazolinone and benzisothiazolinone using a modified local lymph node assay. *Br J Dermatol* 2016 [Epub ahead of print] doi: 10.1111/bjd.14825.

5. CONSIDERATIONS ON METHODOLOGY

This section elaborates the methodology not covered or only briefly described in Manuscripts I–IV.

5.1 Retrospective observational study – Manuscript I

A retrospective observational study was performed to determine the temporal trend of contact allergy to preservatives from the European baseline series and extended series from 1985–2013.

5.1.1 Study design and analyses

So-called surveillance data of contact allergy (from retrospective observational studies) is of utmost importance in the registration of national and regional prevalence ratios of contact allergy in order to monitor potential breaches of Article 3 after post-marketing of cosmetic substances such as preservatives (133, 136, 137). However, inherent limitations in observational studies exist. One major pitfall is that causality can be mistakenly found. Our analyses were primarily descriptive but also included test for association.

'Preservative (contact) allergy' was defined as contact allergy to preselected preservatives from the European Baseline Series and Extended Series. The overall prevalence ratio of contact allergy to preservatives across the test years should be interpreted with caution as the following preservatives were added to the patch-test series during the test period: Iodopropynyl butylcarbamate from 1996, methyldibromo glutaronitrile from 1999, methylisothiazolinone from 2005 and the formaldehyde releasers (2-bromo-2-nitropropane-1,3-diol from 1994; diazolidinyl urea from 1994; DMDM hydantoin from 1994; imidazolidinyl urea from 1994). This will obviously increase the prevalence ratio across the test period. Nevertheless, it is in accordance with our overall conclusion in the manuscript that every time a new preservative is marketed, the prevalence ratio of preservative contact allergy and the overall burden of disease increase due to daily use of cosmetic products. The question to address is, when is the prevalence ratio of newly introduced preservatives acceptable regarding the total number of individuals with contact allergy to a specific preservative?

Furthermore, we used a binary logistic regression model to estimate the impact of variables from

the MOAHLFA index on the dependent variable of 'preservative contact allergy'. Although atopic dermatitis and facial dermatitis were registered only as variables in the database from 1994 and onwards (MOAHL index) and 2001 and onwards (MOAHLFA-index), respectively, we used them in our binary logistic regression model of data for 1985–2013. It is also important to emphasize that 'A' (atopy) in the MOAHL index included 'atopic eczema, allergic rhinoconjunctivitis and/or allergic bronchial asthma', whereas 'A' (atopic dermatitis) in the MOAHLFA index included only atopic dermatitis (140). Another more appropriate statistical approach would therefore have been to include data for all variables and dependent variables only from 2001 and onwards in the binary logistic regression model.

In the analyses, we did not compensate for the 'multiple comparisons problem'. We included 7 variables, all from the MOAHLFA index: 'male sex', 'occupational contact dermatitis', 'atopic dermatitis/atopy', 'hand dermatitis', 'leg dermatitis', 'facial dermatitis' and 'age>40'. The risk of rejecting a 'true' null hypothesis, making a type-1 error ('false positive'), increases with the number of variables. By testing multiple null hypotheses (n=7), the likelihood increases of getting a significant p-value by chance. A few tests exist to compensate for that chance, to control the family-wise error rate, i.e. Bonferroni correction, Holm-Bonferroni method and the Dunn-Šidák correction. Although the original Bonferroni is undoubtedly the easiest test to use, it tends to give a less conservative correction than do the other two. The Boneferroni correction compensates by testing the significant level at a lower level, a level that takes the number of variables into account by setting the new significant level to α/m, where α equals preselected significance level and m number of variables in the analysis. In our analysis, this would give a new significance level of 0.05/7 = 0.0071. With this in mind, we can reject the association between atopic dermatitis and 'preservative contact allergy' and leg dermatitis and 'preservative contact allergy' (Table 1 in Manuscript I). However, we can still conclude that 'preservative contact allergy' is associated with female sex, hand dermatitis, facial dermatitis and age>40. We also showed that MI contact allergy contributed to the increasing number of patients with facial dermatitis in the group of patients with 'preservative contact allergy'. It has previously been shown by binary logistic regression modelling that facial dermatitis is associated with MI contact allergy (3, 13).

5.1.2 Study population and diagnosis of contact allergy

The labour market of the capital region of Denmark has long been characterized by occupations involved in service and administration, craftsmanship, healthcare sector and pharmaceuticals with mainly administrative personnel. In other parts of Denmark, heavy industry and production facilities are often more frequent. This accords with the initial cases of contact allergy to MI being found at production facilities (16, 84).

The patch testing was standardized following the guidelines recently drawn up by the European Society of Contact Dermatitis (116). In Manuscript I, the relevance of contact allergy to MI or methyldibromo glutaronitrile was defined as 'a current and certain association between contact allergy and the allergic contact dermatitis'. In detail, the patients should have current contact with products containing the ascertained allergen. The accuracy of relevance comes down to the systematic exposure assessment, which is time-consuming and safety data sheets may even be inaccurate (141). We included relevancies only from 1999 and onwards; accordingly, we are certain that the majority of relevant cases of contact allergy to methyldibromo glutaronitrile and MI primarily have been registered, for example, due to the mandatory ingredient labelling of cosmetic products in the EU (47, 133). While the use of methyldibromo glutaronitrile in chemical products for occupational use in Denmark is negligible, the use of MI is probably widespread (86, 121). In more recent years, the stepwise systematic exposure assessment has been prioritized and formalized at Herlev-Gentofte University Hospital (141).

The relevance of MI in Manuscript I and Manuscript IV is comparable, and the same definition of relevance is applicable in Manuscript IV.

We emphasize that Thyssen et al. previously have published data from Manuscript I (1985–2008) (45).

5.2 Experimental study – Manuscript II

To estimate the use of MI, MCI, and BIT in paint on the European market, we conducted an experimental study based on paint purchased from five European countries.

5.2.1 Purchase of paints

In each country, a co-author acted as 'person responsible' in the purchase of the paints. All paints were randomly chosen. In the protocol it was stated that each participating country should contribute with 10 white wall paints and 10 wet room paints intended for paint in humid environments. All paints were to represent a broad selection of those on sale in the country, e.g. at retail outlets in, or in the vicinity of, the city: Denmark (Copenhagen), France (Strasbourg), Germany (Erlangen), the United Kingdom (London) and Sweden (Stockholm). Paints were then sent by post/courier to Denmark for analysis. All paint tins were sealed at the time of arrival. A total of 71 tins of paint were sent to the Department of Environmental Science, Aarhus University. Despite our setup stipulating that all five countries should contribute with 20 tins of paint, only a few countries contributed sufficiently: Denmark (n=14), France (n=9), Germany (n=9), the United Kingdom (n=18) and Sweden (n=21). Only 19.4% (14/71) of all purchased paints were wet room paints and none came from Germany and the United Kingdom. This may blur our conclusion of no detectable difference in the concentration of MI between wet room paints and white wall paints. However, our study was exploratory and a conservative statistical approach was chosen based on the aforementioned.

5.2.2 The Danish paints

Despite our overall aim to randomly purchase paints, we initially tried to buy the same Danish paints as analysed in a previous study by Lundov et al. 2014 of Danish paints purchased in 2012 (85). However, this was not possible and the approach was dropped during the purchase phase. This prevents us from saying that the Danish paints were randomly purchased. The paints purchased for the initial study by Lundov et al. represented a wide range of brands in Denmark (85). No statistical difference was observed between the countries regarding MI concentration.

5.2.3 Environmental labelling
See Manuscript II for details on environmental labelling of MI and safety data sheets.

5.2.4 Experimental analysis

We used high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (MS-MS) in the experimental analyses of isothiazolinones of all 71 purchased paints. This method is well recognized as an analytical chemistry technique with high sensitivity of quantification of the analyte in complex mixtures such as paint (24). The precision of the method was calculated as the relative standard deviation of replicate analysis of 12 pairs. In our study, it was 1.3% for MI and 1.5% for BIT. This is in accordance with the previous study by Lundov et al. (85).

There are other ways of isolating analytes, for example, gas-liquid partition chromatography (GLPC), but we chose HLPC due to the sensitivity and safety during the isolation of the analyte with no substantial risk of decomposition of the analyte.

5.2.5 Octylisothiazolinone

Although occupational relevant OIT contact allergy was recognized in painters in 2012/13, we included only MI, MCI and BIT for detection in our purchased water-based paints (61, 112). At the time of the purchase and analyses of the paints, we did not realize the potential importance of co-sensitisation and/or cross-reactivity between MI and OIT (3, 112).

Currently, the National Allergy Research Centre is conducting analyses of newly purchased European water-based paints. Here, we found that only 27.6% (16/58) of the experimentally analysed paints had detectable amounts of OIT (Median 0.51 ppm; IQR 0.21-4.80). These low concentrations are not in accordance with a mean concentration of 177 ppm for OIT registered in the Danish Product Register (86). However, the data registered in the Danish Product Register cannot be used to conclude in what concentration OIT is found in the final paint product (86). Other preservatives may also have been of interest, for example, other isothiazolinones or the current use of formaldehyde in water-based paint, but this was outside the scope of the current study (96, 142).

5.3 Animal study – Manuscript III

To investigate the potential cross-reactivity between MI and OIT, and MI and BIT, we conducted

an experimental study based on the modified LLNA with the aforementioned isothiazolinones.

5.3.1 Groupings

All groupings included 8–9 mice based on two independent experiments on different days. During the phases of sensitisation and elicitation, we used relatively high concentrations of MI, OIT and BIT (their EC3 values). Lower concentrations of MI would require more and frequent exposures to elicit the same immune response, which would unnecessary stress the mice. This is an acceptable approach when testing allergenic potential of an allergen, but it may differ regarding humans and does not necessarily mimic the exposures humans experience: repeated and long-lasting exposure to the same cosmetic product containing the specific allergen.

It would have been beneficial to include a 'positive control': mice sensitised with MI in the three different concentrations and challenged with OOA. This is a notable limitation. Instead, as stated in our protocol, we exposed mice with OOA during the phase of sensitisation and challenged them with OOA or 0.4% MI.

5.3.2 Purity analysis of the used standards

Notably, we found that cross-contamination was negligible because purity analyses showed that analytes of MI did not contain BIT and OIT and *vice versa*. Impurities were later calculated based on stock solution concentration and detection limit (Table 1 in Manuscript III). Accordingly, to the best of our knowledge, we can reject the notion that the mice were sensitised to BIT and OIT during the phase of sensitisation or that MI-sensitised mice were exposed to MI when being challenged with BIT and OIT.

5.3.3 EC3 values

An inverse correlation exists between the sensitisation potential of an allergen and the EC3 value: the lower the EC3 value, the more potent is the allergen regarding sensitisation. In Manuscript III, we used established EC3 values for MI and BIT, whereas the value for OIT was estimated because no value has been published (60, 143-145). The EC3 value of MI has previously been erroneously quoted (moderate sensitiser), but since 2013 it has been recognized as a strong sensitiser with an

EC3 value of 0.4% (60, 133, 144).

5.3.4 Estimation of EC3 value for octylisothiazolinone

OIT was regarded as a strong sensitiser with an estimated EC3 value of 0.7%. Here, we assumed that the MI and homologues' potency was not logP dependent. Molar potency would then be regarded as the same and in terms of weight %, it would depend on molecular weight due to the octyl homologue (Table 1). Given these assumptions, the EC3 value for OIT is 0.7% as the molecular weights divided by each other multiplied by the EC3 value for MI give: 213/115 * 0.4 = 0.7.

However, the estimation of the EC3 value for OIT contains some uncertainties. If the potency of MI and OIT are considered logP dependent, the potency of OIT will increase by a factor 33. For mechanistic domains where logP dependency has been shown and QMMs have been developed (SB domain and SN2 domain) the logP coefficient in equations correlating EC3 is about 0.4. A CH2 group contributes about 0.54 logP units, so the difference in logP between OIT and MI will be about 3.78 (7*0.54 = 3.78)(Table 1). Multiplying by the assumed QMM coefficient of 0.4 and taking the antilog, the logP effect would be to increase the potency by a factor 33, given OIT, EC3 = 0.02%, an extreme sensitiser.

In our study, OIT was considered a strong sensitiser (EC3 = 0.7%) instead of an extreme sensitiser (EC3 = 0.02%). Under this assumption, we tested our hypothesis of cross-reactivity between MI and OIT. However, if OIT is to be considered an extreme sensitiser and we challenged the MI-sensitised mice with a concentration of 0.7% OIT, the concentration would be 35 times too high and may hinder any firm conclusion based on the results. The immunological response in the draining lymph node is concentration dependent and the advantage of the EC3 value is that it is the exact concentration of a chemical that gives a threefold increase in the number of cells in the draining lymph node [Gerberick 2007]. A concentration that is 35 times the EC3 value may increase the expected EC3 value response many fold and thereby give an increased immunological response in the groups challenged with 0.7% OIT.

Further, another, more time-consuming approach would be to experimentally determine the EC3

value of OIT by the LLNA.

5.3.5 Hypotheses, statistical significance and power

In our null hypothesis, we hypothesized that the same immunological response would be mounted when MI-sensitised mice were challenged with MI, OIT or BIT. In the alternative hypothesis, we hypothesized is that the immunological response MI-sensitised mice will mount when being challenged with MI, OIT or BIT differs. As stated, we did not include a control group of MI-sensitised mice challenged with a vehicle.

Statistical significance was defined as $p<0.05$. The power is defined as the probability that the test correctly rejects the null hypothesis. The risk of a Type II error (false negative) decreases with increasing power. The power is based on (i) the chosen significance level ($p=0.05$), (ii) effect size in the population (here immunological response), and lastly (iii) the sample size (n=8-9). Two ways to increase the power would be to increase the number of mice or to reduce measurements errors. Prior to the study, we did not conduct *a priori* power analysis as this approach is uncommon for murine studies and we choose n=8-9, which is an accepted approach, both ethically and scientifically.

However, *post-hoc* power analysis is not appropriate due to its controversy. Another more suited approach is to consider the 95% CI in order to have a surrogate for power; those we have already created (and preselected) for Figure 4 in Manuscript III. In figure 4, relatively narrow 95% CI is overlapping, indicating that the same immunological response was mounted when MI-sensitised mice were challenged with MI, OIT or BIT.

5.4 Prospective observational study – Manuscript IV

In Manuscript IV, we aimed to investigate MI contact allergy based on a European multicentre study of prospectively collected data during May 2015–October 2015.

5.4.1 Study design and analyses

Observational prospective studies tend to be superior to retrospective studies. This study, with prospectively collected data, is the first to elucidate the widespread epidemic of MI contact allergy

across several European countries. Only consecutive patch-tested patients were included, thereby we avoid mistakenly found causality in, for example, cohorts based on aimed patch-tested patients with patients being patch tested with only the metal fluid series or hairdresser series. All centres were general centres of dermatology, apart from one. Despite the FIOH (the Finish Institute of Occupational Health) in Finland being a centre specializing in occupational cases, it contributed only 3.4% (7/205) of all patients with MI contact allergy. However, it was in the FIOH that the highest prevalence ratio of MI contact allergy was registered (13.0%; 7/54). In general, the centres that contributed with data were located across both the Northern (n=165) and Southern EU (n=40) (Fig. 3). Other studies have focused mainly on one country/region (3, 5, 6, 8-10, 12-14). .

Figure 3. Geographical location of the centres contributing data on 205 patients with contact allergy to methylisothiazolinone.

5.4.2 Considerations on ethics

No ethics approval for this prospective observational study was needed according to the Local Human Ethics Committee. However, all patients gave written informed consent before inclusion. The Data Protection Agency approved storage of unanonymised data from Copenhagen University Hospital Gentofte (Journal number: GEH-2015-076 and I-suite number 03709). All other collaborators followed their regional and/or national guidelines for storage of patient data. All data were anonymised at each European centre before being sent to the National Allergy Research Centre (see supplemental Table 1 in Manuscript IV).

5.4.3 Patch testing across European countries and diagnosis

Some variance in test systems and producers existed across all countries in Manuscript IV (Table 1 in Manuscript IV). We do not believe this influenced the patch-test results. We included only patients with a positive patch-test result to 2000 ppm MI aq. (reactions designated as +,++,+++). There is an inherent risk of measurement bias regarding a positive patch-test result of MI, but all centres followed the most recent European recommendations on patch testing (116). However, not all patients had patch-test readings performed on D2, D3/4 and D7 according to this guideline (116). Six patients had their (positive) readings performed only on D2 and D7 with no difference between the two readings, apart from one patient with a weak positive reaction (+) on D2 compared with a strong reaction (++) on D7. Only one patient had a positive reaction (+) on D2, a doubtful reaction on D3/4 and a negative reaction on D7. Two patients had no patch-test reading done on D2; they had a negative and doubtful reaction on D3/4, respectively, and a positive patch-test reaction (+) on D7. Theoretically, some patients may not be included in the study because they have been overlooked (116).

In the manuscript, polysensitisation was defined as the presence of contact allergy to three or more unrelated allergens (146). A post-hoc analysis showed that 24.9% (n=51) of MI positive patients were polysensitised. However, only selected allergens from the European Baseline Series were predetermined on the form (supplementary Table 1 in Manuscript IV); this may have resulted in an underestimation as only these frequent, predetermined allergens may have been registered. The ratio of polysensitisation should be interpreted with caution in Manuscript IV as it

was only a secondary aim in the study with methodological limitations.

The diagnosis of widespread contact dermatitis was defined as dermatitis at more than three anatomical sites. The anatomical sites were preprinted and included the following: hands, arms, face, scalp, eyelids, neck, trunk, anogenital area, legs and feet. 'Widespread' refers to a large percentage of the skin being involved, here with dermatitis. Our definition is somewhat contradictory as we counted all 10 anatomical sites as actual anatomical sites, but it could be argued that the dermatitis is not widespread if it is localized to only the eyelids, face, scalp and the neck. Nevertheless, no patients were affected by dermatitis only at these sites. No formal definition exists on generalized dermatitis.

6. DISCUSSION

Discussion of the results from Manuscripts I–IV in more general terms.

6.1 Recurring epidemics of contact allergy to preservatives – Manuscript I

Unfortunately, epidemics of contact allergy to specific preservatives develop as shown in Manuscript I and by others (3, 6, 13, 40, 42, 44, 45, 98). We found prevalence ratios of 4–6% for methyldibromo glutaronitrile and MI during 1999–2013. A novel finding was that the prevalence ratio of methyldibromo glutaronitrile continues to be high, but with decreasing relevance, even after the ban of methyldibromo glutaronitrile in cosmetic products.

The use of methyldibromo glutaronitrile is low in chemical products for occupational use (142). Methyldibromo glutaronitrile was registered in only four products in the Danish Products Register Database (PROBAS) in 2014; however, there is no specification due to confidentiality. In comparison, MI was registered in 830 products out of 38,000 active substances and materials (142). This observation may partly explain the significant decrease in relevance of contact allergy to methyldibromo glutaronitrile to <10% after its use in cosmetic products was banned. Nevertheless, other retrospective studies have found decreasing prevalence ratios of methyldibromo glutaronitrile shortly after the ban (41, 43, 130). A Danish retrospective study of

19 279 consecutive patch-tested patients from the Danish Contact Dermatitis Group concluded that the prevalence ratio of methyldibromo glutaronitrile contact allergy significantly decreased from 4.6% in 2003 to 2.6% in 2007 (41). The current relevance of methyldibromo glutaronitrile was also observed to decrease from 51.3% in 2003 to 29.0% in 2007 (41). In the study, the number of centres (tertiary clinics and dermatologists in private practice) increased over the test years (41). In our study, we also observed a decline in the prevalence ratio of contact allergy to methyldibromo glutaronitrile from 2003 to 2007, but with an increasing prevalence ratio of methyldibromo glutaronitrile from 2007 to 2010, a decline from 2010 to 2012, and an increase from 2012 to 2013 (Fig. 1 in Manuscript I). Although some variance across test years will always be found, we did not find any significant decrease/increase in the prevalence ratio of contact allergy to methyldibromo glutaronitrile. Additional analyses of the data, not published in Manuscript I, show that patients with methyldibromo glutaronitrile contact allergy have a higher frequency of contact allergy to formaldehyde (7.7%) and contact allergy to MI (14.6%). This is in accordance with the observed decreasing relevance of methyldibromo glutaronitrile, for example, in a patient with suspected allergic contact dermatitis where the patch-test results show a relevant MI contact allergy and an irrelevant methyldibromo glutaronitrile contact allergy with former relevancy. Further, in Lithuania, methyldibromo glutaronitrile contact allergy was found in 3.7% of consecutive patch-tested patients tested during 2014–2015 (147). In comparison with patch-test results from the same centre from 2006–2008, the prevalence ratio of methyldibromo glutaronitrile contact allergy was stable (5.5%) (147, 148).

A comprehensive European multicentre study recently showed that methyldibromo glutaronitrile contact allergy was predominantly found in patients with older age after stratification into the age groups '16–64*yrs*' and '>64*yrs*' (up to 2.9 and 3.7, respectively) (149). However, regional differences were observed, and in the Netherlands, a relatively high frequency of methyldibromo glutaronitrile contact allergy was observed (6.9%) compared with that in the UK (0.6%) (149). Despite the majority of all cases of methyldibromo glutaronitrile contact allergy being observed in the two oldest age groups, patients younger than 16yrs of age also had the allergy [Giménez Arnau 2016]. This may be the result of early sensitisation to methyldibromo glutaronitrile (before 2008) for the 'oldest' patients in the age group '<16yrs' or by non-regulated sources, such as a preservative in topical medicaments, where methyldibromo glutaronitrile is not necessarily

declared (149, 150).

Notably, we found a relevance of MI contact allergy of 60–80% across the test years 2005–2013, but after even a complete ban on MI in leave-on cosmetic products and a restriction in rinse-off cosmetic products, patients with unrecognized MI contact allergy may still be exposed to MI from consumer products such as water-based paint. The high ratio of relevance may indicate that the epidemic of MI contact allergy was still gaining pace in 2013 with new cases of allergic contact dermatitis due to exposure to products containing MI. However, Manuscript I did not include any exposure analysis.

'Preservative contact allergy' was defined as contact allergy to preselected preservatives from the European Baseline Series and Extended Series, but since the definition varies over time due to the introduction of new preservatives in the series there are some limitations that must be addressed: (i) preservatives are largely different chemicals with individual capability to preserve and sensitise, (ii) the introduction of all new preservatives in the patch-test series does, nonetheless, increase the overall prevalence ratio of contact allergy to preservatives, and (iii) the introduction of new preservatives along with new registered variables (e.g. atopic dermatitis and facial dermatitis) may lead to false deductions.

We found that preservative contact allergy was associated with facial dermatitis. However, facial dermatitis was not registered systematically in the MOAHLFA index until 2001, in contrast to the prevalence ratio of preservative contact allergy that steadily and significantly increased over the test years. The conclusion based only upon the logistic regression model of all patients may therefore falsely draw an association with preservative contact allergy and facial dermatitis because the premises are different. Looking only at the data from 2001 when facial dermatitis was systematically registered and onwards showed that facial dermatitis affected 20–25% during 2001–2009. Thereafter a steep and significantly increase in the frequency of facial dermatitis was observed, mainly driven by the new cases of MI contact allergy. Further, we estimated MI contact allergy adjusted attributable risk percentages with facial dermatitis that was 40% and 49% for 2010–2013 and the year 2013, respectively. This adds to the overall interpretation of the data that MI contact allergy affects the anatomical region of the face, as we also showed in Manuscript IV

where 32.7% of all patients with MI contact allergy had facial dermatitis.

6.2 Methylisothiazolinone is widely used in water-based paint – Manuscript II

The use of MI in chemical products for occupational use is diversified and according to a Danish experimental study, MI is often added to Danish water-based paints (85, 86). In agreement with this, we experimentally showed that MI is extensively used in water-based paint in high concentrations across five European countries. Additionally, we showed that BIT was also used extensively in high concentrations. Lastly, we showed that, in many cases, the labelling of MI is insufficient and that environmental labels do not protect the European consumer sufficiently regarding exposure to MI.

Our data are in accordance with several cases showing that patients may experience allergic contact dermatitis due to skin contact or airborne contact with evaporated MI from MI-preserved water-based paint (46, 77, 92-94, 151-156). Taken together with our data in Manuscript IV where approximately 7% had experienced allergic symptoms whenever entering a newly painted room, this calls for action.

One way of avoiding elicitations in patients who already have MI contact allergy is by labelling the products that contain MI and it is pivotal that MI is labelled correctly. MI must be labelled only if the concentration exceeds 1000 ppm. In general, chemicals not classified as a skin sensitiser in the EU must be labelled on the product and on the safety data sheet only if the concentration exceeds one-tenth of the standardized 'generic concentration for classification and labelling' of 10,000 ppm (1%) according to the rules of self-classification (90) (90). All water-based paints in Manuscript II contained MI concentrations well below 1000 ppm and should therefore not be labelled according to the rules of self-classification.

After several years of delay, the Committee for Risk Assessment (150) concluded that MI should be classified as 'Skin Sens 1A, H317', with a specific concentration limit of 0.0015% (89). The final decision on the abovementioned recommendations is still awaited and will be decided in spring 2017.

Initially, a specific concentration limit of 0.06% (600 ppm) was proposed, but after a public hearing this concentration was considered too high to sufficiently protect the European consumer against MI contact allergy and allergic contact dermatitis due to exposure to MI in chemical products for industrial use, including water-based paint (89). Until MI is classified as a skin sensitiser, the

manufacturers can legally omit information regarding the content of MI in their chemical products for occupational use, providing the rules of self-classification according to the regulation on classification, labelling and packaging of substances and mixtures (CLP Regulation) are adhered to (90). Consequently, the safety data sheets and paint tins registered in Manuscript II did not state the content of MI in the products. Without correct labelling of MI, it is impossible to avoid exposure unless the manufacturers willingly state its content. In the future, after final implementation of the CRA recommendations to classify MI as a skin sensitiser and according to the CLP, manufactures will be obliged to state the content of MI if the concentration exceeds one tenth of the specific concentration limit of 15 ppm (1.5 ppm, the so-called 'concentration limit for elicitation') (90).

However, MI labelling of a product such as water-based paint is mostly for the benefit of consumers who know they have an MI contact allergy and who can recall this when purchasing the product. However, no questionnaire studies have been conducted in patients with MI contact allergy. A questionnaire study in patients with chlorhexidine contact allergy showed that after their diagnosis, 32% had experienced accidental exposure to products containing chlorhexidine, and that only 38% and 83% were aware of the use of chlorhexidine in cosmetic products and hospital/dentist settings, respectively (157). Patients with different preservative contact allergies are probably equally well or badly equipped to manage their contact allergy. After the regulation of MI in cosmetic products, the use of MI in chemical products for occupational use may persist, posing a risk of accidental flare-up episodes, for example, by airborne allergic contact dermatitis due to exposure to evaporated MI from water-based paint.

In Manuscript II, we failed to show a statistically significant decline in the dose of MI in water-based paint purchased in December 2014/January 2015 compared with the aforementioned study by Lundov et al. of MI-concentration in Danish water-based paint randomly purchased in 2012 (85). Currently, the National Allergy Research Centre is analysing the content of MI and other isothiazolinones in European water-based paint from the same five European countries as in Manuscript II.

6.3 The potential cross-reactivity between isothiazolinones – Manuscript III

This novel approach to address cross-reactivity between isothiazolinones by a modified local

lymph node assay shows that cross-reactivity between MI and OIT, and MI and BIT may exist. Initially, we showed that mice were sensitised to MI after a sensitisation period and that the preselected EC3 value for MI initiated a threefold cellular response in the draining lymph node. Lastly, we showed that the concentration-dependent challenge response was similar in MI-sensitised mice that were challenged with MI, OIT and BIT.

Initially, we showed the that the sensitising potential of MI in the modified LLNA is in accordance with previous published data showing that MI possesses strong sensitising capability with an EC3 value of 0.4% in an LLNA (60, 144, 158). This is further in accordance with surveillance data in this thesis and previously published work (3, 5, 6, 8-10, 12-14).

Murine studies are preferred when considering cross-reactivity as they show 'maximum scenarios' (0.13% MI, 0.4% MI and 1.2% MI) compared with surveillance data that often lack sufficient knowledge of exposures and assumed co-sensitisation, possibly even when there is none. A notable example comes from us, who once assumed that OIT was to be found in paints and, accordingly, viewed this as a relevant exposure. However, the aforementioned ongoing experimental study of purchased paints does not necessarily verify this picture (86, 112).

In 1996, Geier et al. rejected the notion that cross-reactivity existed between MCI/MI, OIT and BIT (114). In a Danish retrospective study, only 8.8% (15/170) patients with MI contact allergy also had BIT contact allergy, where BIT was patch tested in the concentration 500 ppm aq. or 1000 ppm aq. (62). In the same study, it was found that 44.1% of patients with a positive patch-test result to BIT also had a positive patch-test result to MI 2000 ppm aq. (62). However, this study had some limitations as not all patients included in the analyses were consecutively patch tested with BIT and no data on exposures were available (62). It is therefore unknown whether this was due to co-sensitisation or cross-reactivity between MI and BIT.

In 2014, another retrospective observational study of 6599 patients showed that cross-reactivity between MI and OIT existed (3). However, this conclusion was based on patients who were aimed patch tested with OIT (n_{total} = 199). Fifteen patients had a positive patch-test reaction to MI and OIT, but no relevant exposures to OIT; cross-reactivity with MI was therefore considered likely (3). In 2015, a retrospective observational analysis of 3938 patch-tested patients in Germany further

showed that 8.5% (21/248) and 6.0% (15/248) of patients with MI contact allergy also had OIT contact allergy and BIT contact allergy, respectively (106). Therefore, it was concluded that the observed concomitant patch-test reactions between these isothiazolinones were due to co-sensitisation rather than cross-reactivity (106).

In 2016, a Belgian study showed that two patients with suspected OIT allergic contact dermatitis due to relevant exposure to leather goods also had a positive patch-test reaction to MI with no relevant exposures (111). Further, the authors concluded that the recommended patch-test concentration of OIT of 250 ppm pet. was too low to detect the OIT contact allergy and a patch-test concentration of 1000 ppm OIT pet. was needed (111).

The patch-test dose of OIT (250 ppm) in the extended patch test series may be too low to sufficiently detect OIT contact allergy. The recommended patch test concentration of MI is 2000 ppm aq. whereas the patch test concentration of BIT varies between 500–1000 ppm aq. The patch-test doses of OIT and BIT need to be optimized in same way as has the patch-test dose of MI before observational studies can be considered sound regarding cross-reactivity between isothiazolinones.

Recently, a French comprehensive experimental study investigated the in situ behaviour of MCI and MI. It was concluded that cross-reactivity between MCI (without the MI component) and MI would be unlikely (159). No such studies of in situ behaviour of MI, OIT and BIT have yet been published.

6.4 The prospective European multicentre study – Manuscript IV

This is the first prospective European multicentre study in patients with MI contact allergy. An overall prevalence ratio of 6.0% of MI contact allergy is relatively high and in many cases higher than the prevalence ratios of MI contact allergy observed in previous retrospective observational studies most of these with data for 2010–2013 (3, 5, 6, 8-10, 12-14). The epidemic of MI contact allergy is persisting and still gaining pace, at least based on our data from 2015 in comparison with the retrospective data. However, a recent British surveillance study showed that the incidence of MI contact allergy peaked in 2013 with a minor decrease in 2014 (160). Taken together, our data cannot be used for longitudinal conclusions regarding the development of MI contact allergy; one retrospective study of regional data may also be too few to conclude on plateaus (160). In Leeds

and London, we found relatively high prevalence ratios of MI contact allergy of 5.2% and 5.1%, respectively. The persistent high prevalence ratio of relevant MI contact allergy across European countries shows that the cosmetic industry cannot regulate itself.

Furthermore, we found that the majority of patients with MI contact allergy noted onset of their dermatitis during 2013–2015 and that 72.7% had a current relevancy of their MI contact allergy. This could mean that the pace of the epidemic of MI contact allergy is not decreasing and that citizens across the EU continue to become sensitised by skin exposure to products containing MI.

Notably, the anatomical localizations of the dermatitis-affected body parts are often exposed to cosmetic products (incl. wet wipes): hands, face, eyelids, neck and ano-genital region. An alarming 12.7% of the patients had widespread dermatitis, which may indicate the severity of their contact allergy. To date, the Quality of Life (QoL) or Dermatology Life Quality Index (DLQI) in patients with MI contact allergy has not been reported, but it is well known that life quality is impaired in dermatitis patients, for example, in individuals with contact allergy to fragrances or those with occupational contact dermatitis (161, 162).

Polysensitisation and simultaneous contact allergy to fragrance mix I, fragrance II and formaldehyde in patients with MI contact allergy were in accordance with other studies (146, 163). However, it is important to emphasize that this was a secondary comparison and further research in polysensitisation of patients with MI contact allergy is needed before any firm conclusions can be drawn.

Exposure analyses revealed that the exposure to MI-containing leave-on and rinse-off cosmetic products accounted for 83.2% of all patients with relevant MI contact allergy. The first retrospective published study showed that 100% of patients with MI contact allergy were exposed to MI contained in cosmetic products, primarily due to the use of wet wipes (46). Later retrospective studies of consecutive patients showed that relevant MI contact allergy is increasing primarily due to exposure to cosmetic products (incl. wet wipes), which accounts for approximately 60–70% of relevant cases of MI contact allergy (3, 13, 69).

In Italy, a recent retrospective observational study showed that rinse-off cosmetic products and household products were relevant exposures in patients with MI contact allergy (75). This is in

accordance with our data in Manuscript IV, as 58.4% (87/149) of patients with relevant MI contact allergy were exposed to rinse-off cosmetic products either alone (38.9%; n=58) or in combination with leave-on cosmetic products (19.5%; n=29).

This should certainly be taken into account if and when the EC considers implementing the suggested restriction of MI in rinse-off cosmetic products by the SCCS (63, 64, 67, 68). Since December 2013, the trade organization Cosmetics Europe has advised their members to discontinue the use of MI in leave-on cosmetic products in the interests of consumer safety; however, our data reveal that MI is still found in many different brands and manufacturers (164). This could be because retail stores are still selling off already manufactured leave-on cosmetic products, but it is more likely due to the continued use of MI in leave-on cosmetic products. Further, in 2015 in a comprehensive ROAT study with 2 liquid hand soaps preserved with MI at 50 ppm and 100 ppm, Yazar et al. showed that the use of MI in rinse-off cosmetic products elicits allergic contact dermatitis in patients with MI contact allergy (65). This is in accordance with our data revealing that 38.9% of patients with relevant MI contact allergy were exposed only to MI contained in rinse-off cosmetic products.

Initial steps have been taken to implement the advice of the SCCS to restrict the use of MI in rinse-off cosmetic products. In spring 2016, a public consultation on MI in rinse-off cosmetic products was launched, ending 1 July 2016 (67, 68). The conclusion has yet to be published. Despite MI in leave-on cosmetic products being fully restricted from February 2016 and initial steps being taken to restrict MI in rinse-off cosmetic products, the cosmetic market may continue to sell off already manufactured leave-on cosmetic products containing MI, and MI may still be used in rinse-off cosmetic products at a maximum concentration of 100 ppm.

7. CONCLUSIONS AND PERSPECTIVES FOR FURTHER RESEARCH

7.1 The epidemic of contact allergy to methylisothiazolinone and the failed risk management process

The unprecedented epidemic of contact allergy to MI on the European continent has raised awareness of the risk assessment procedure in the EU. In Manuscript IV, we saw that the

prospectively registered prevalence ratio of MI contact allergy was 6.0% (range 2.0% to 13.0%), which is in accordance with Manuscript I and several retrospective studies across Europe (3, 5, 6, 8-10, 12-14). However, our study is the first and only prospective European study that shows high prevalence ratios of contact allergy to MI across European countries.

Further, in Manuscript IV we suggest that restrictions on the use of MI in rinse-off cosmetic products are necessary because 58.4% (87/149) of the patients with a relevant MI contact allergy had had skin contact with an alarmingly high number of MI-containing rinse-off cosmetic products. Our study does not necessarily say anything about the risk of sensitisation when being exposed to rinse-off cosmetic products containing MI. A total of 38.9% (58/149) of our patients with a relevant MI contact allergy possessed MI-containing rinse-off cosmetic products, and it is therefore possible that these patients have been sensitised purely due to skin contact with rinse-off cosmetic products containing MI.

In Manuscript I, we suggested that the European risk assessment and risk management process of cosmetic ingredients should be changed based upon the presence of the current epidemic of contact allergy to MI. While the pre-market risk assessment process of MI in 2003–2005 was regarded as sufficient with no risk to the European consumer, the post-market surveillance data of contact allergy to MI showed that the use of MI is a burden for the general health of the European consumer regarding contact allergy. Since this recognition in 2010 and onwards, scientists and national healthcare and environmental authorities have tried to raise awareness of the troublesome use of MI in cosmetic products in the EU (7, 98). However, they have had little success; MI for use in leave-on cosmetic products was not banned until February 2017 and the use of MI in rinse-off cosmetic products awaits legal measures by the EC after it has been recommend by the SCCS to limit its use in rinse-off cosmetic products to 15 ppm (63, 64, 67, 68).

The current EU Cosmetic Products Regulation states that 'in substances which are likely to cause allergy to a significant part of the population, other restrictive measures such as a ban or a restriction of concentration should be considered' (133). In view of this and the insufficient, and untimely risk management of MI by the EC, we proposed in Manuscript I (and later modified) another approach to equip the EC with a timely remedy to legally address and withdraw the use of troublesome substances in cosmetic products in the EU to avoid full-blown epidemics (165). Substances are currently granted time-unlimited entry into Annex V, that is, substances that may

be used in cosmetic products in the EU (see introduction) (133, 165). Instead, we propose that all new substances, with an adequate and positive opinion by the SCCS, should be granted only time-limited entry into the Annex V instead of the current time-unlimited entry (121). Accordingly, surveillance data and real-life experience of the substance can be taken into account before the substance is granted unlimited entry into the Annex V (121). In all its simplicity, we hope that future delays can be avoided in risk management of troublesome substances allowed for use in cosmetic products.

7.2 The use of methylisothiazolinone in paint

The extensive use of MI in European water-based paint calls for action. Not only painters occupationally exposed to water-based paint or European citizens who paint their homes are at risk, but also citizens who are unknowingly exposed to evaporated MI from newly painted walls. Approximately 7% of the European patients with MI contact allergy in Manuscript IV had experienced allergic symptoms when being in newly painted rooms, which indicates that future studies should focus on whether MI continues to be used in water-based paint purchased in Europe. Interest groups and media have tried to raise awareness of the alarmingly high concentrations of MI in water-based paint. The proposed classification of MI as a skin sensitiser with a specific concentration limit of 15 ppm is on the right track, but a total ban of MI in water-based paint is advisable when considering the number of patients with MI contact allergy in the EU and the high domestic use of water-based paint. Overall, in accordance with our data in Manuscript II, it is possible to preserve water-based paint with a low content or no content of MI (and BIT).

7.3 Cross-reactivity between isothiazolinones

Taken together, we showed that cross-reactivity between MI and OIT, and MI and BIT may exist because the same immunological response was observed in MI-sensitised mice challenged with MI, OIT and BIT. There is always a question of whether another study design would have been more appropriate to test for cross-reactivity, for example, an additional control or in situ behaviour of MI, OIT and BIT based on the reconstructed human epidermis or even observational

studies (3, 6, 106, 159). Different conclusions have also been drawn from different study designs. Experimental studies under standardized conditions may, however, ethically and study-wise be the best approach and be superior to other designs. Further, in the light of the epidemic of MI contact allergy, we emphasize that a conservative approach regarding the potential cross-reactivity between MI and isothiazolinones in general should be considered when future legislation is drawn up on the use of OIT and BIT in cosmetic products.

In 2012, the SCCS advised the EC against the use of BIT in cosmetic products due to the ongoing and rapid increase in cases of MI contact allergy in the EU (117). Since then the epidemic of MI contact allergy has been fully recognized and the final legislative steps on curbing the epidemic have been initiated. The absolute number of recognized and un-recognized European citizens with MI contact allergy will persist and will be alarmingly high for years if the epidemic of MI contact allergy follows the same pattern as the epidemic of methyldibromo glutaronitrile contact allergy with a high prevalence ratio but decreasing relevance (Manuscript I). It is plausible that a great number of the European citizens with recognized and un-recognized MI contact allergy may experience cross-reactivity to OIT or BIT and elicitation of allergic contact dermatitis if these preservatives are used in cosmetic products. Based on our data, we therefore highlight that the EC is obliged to be a commendable risk assessor and risk manager and should not permit OIT and BIT in cosmetic products (121).

In conclusion, this thesis concerning contact allergy to methylisothiazolinone contributes with the following novel observations:

- The prevalence of contact allergy to MI has increased rapidly in a Danish tertiary clinic since 2010 and has contributed significantly to an increasing prevalence of facial dermatitis in patients with preservative contact allergy.

- MI is widely used in water-based paint in five European countries in high and troublesome concentrations. This observation highlights the need for intervention on the use of MI in European water-based paint.

- Cross-reactivity between MI and BIT, and MI and OIT was detected in our modified LLNA. We therefore emphasize that this novel finding should be considered in future risk assessment of OIT and BIT.

- The use of MI in cosmetic products across eight European countries has contributed to the unprecedented epidemic of contact allergy to MI in these countries. Nearly an eighth of the patients had severe, more widespread dermatitis due to exposure to MI-containing cosmetic products. The restriction of MI in leave-on cosmetic products in the EU is in place, but legislative restriction of MI in rinse-off cosmetic products awaits (necessary) action based upon our novel findings. The use of rinse-off cosmetic products containing MI contributed solely to 38.9% of all patients with relevant contact allergy to MI.

8. References

1 Rustemeyer T, Von Hoogstraten IMW, von Blomberg BME, Gibbs S, Scheper RJ. Mechanisms of Irritant and Allergic Contact Dermatitis. In: Contact Dermatitis, 5th Edition. Johansen JD, Frosch PJ, Lepoittevin J-P (Eds.). Berlin Heidelberg, Springer-Verlag, 2011: 43-90.

2 Diepgen TL, Ofenloch RF, Bruze M, Bertuccio P, Cazzaniga S, Coenraads PJ, Elsner P, Gonçalo M, Svensson A, Naldi L. Prevalence of contact allergy in the general population in different European regions. *Br J Dermatol* 2016: **174**: 319-29.

3 Aerts O, Baeck M, Constandt L, Dezfoulian B, Jacobs MC, Kerre S, Lapeere H, Pierret L, Wouters K, Goossens A. The dramatic increase in the rate of methylisothiazolinone contact allergy in Belgium: a multicentre study. *Contact Dermatitis* 2014: **71**: 41-8.

4 Bruze M, Gonçalo M, Lepoittevin J-P, Diepgen T, Orton D. Incompetence and Failure to Regulate Methylisothiazolinone. *Contact Dermatitis* 2015;72:353-4.

5 Gameiro A, Coutinho I, Ramos L, Goncalo M. Methylisothiazolinone: second 'epidemic' of isothiazolinone sensitization. *Contact Dermatitis* 2014: **70**: 242-3.

6 Geier J, Lessmann H, Schnuch A, Uter W. Recent increase in allergic reactions to methylchloroisothiazolinone/methylisothiazolinone: is methylisothiazolinone the culprit? *Contact Dermatitis* 2012: **67**: 334-41.

7 Gonçalo M, Goossens A. Whilst Rome burns: the epidemic of contact allergy to methylisothiazolinone. *Contact Dermatitis* 2013: **68**: 257-8.

8 Hosteing S, Meyer N, Waton J, Barbaud A, Bourrain J L, Raison-Peyron N, Felix B, Milpied-Homsi B, Ferrier Le Bouedec M C, Castelain M, Vital-Durand D, Debons M, Collet E, Avenel-Audran M, Mathelier-Fusade P, Vermeulen C, Assier H, Gener G, Lartigau-Sezary I, Catelain-Lamy A, Giordano-Labadie F. Outbreak of contact sensitization to methylisothiazolinone: an analysis of French data from the REVIDAL-GERDA network. *Contact Dermatitis* 2014: **70**: 262-9.

9 Johnston GA, Contributing Members of the British Society for Cutaneous Allergy (BSCA). The rise in prevalence of contact allergy to methylisothiazolinone in the British Isles. *Contact Dermatitis* 2014: **70**: 238-40.

10 Lammintausta K, Aalto-Korte K, Ackerman L, Alanko K, Berry P, Hasan T, Kaminska R, Korhonen L, Laukkanen A, Liippo J, Pesonen M, Rantanen T, Riekki R, Suuronen K. An epidemic of contact allergy to methylisothiazolinone in Finland. *Contact Dermatitis* 2014: **70**: 184-5.

11 Lundov MD, Thyssen JP, Zachariae C, Johansen JD. Prevalence and cause of methylisothiazolinone contact allergy. *Contact Dermatitis* 2010: **63**: 164-7.

12 Madsen JT, Andersen KE. Further evidence of the methylisothiazolinone epidemic. *Contact Dermatitis* 2014: **70**: 246-7.

13 Uter W, Geier J, Bauer A, Schnuch A. Risk factors associated with methylisothiazolinone contact sensitization. *Contact Dermatitis* 2013: **69**: 231-8.

14 Warburton KL, Wilkinson M. Contact allergy to methylisothiazolinone: Has there been any change? Experience of a UK centre. *Contact Dermatitis* 2015: **72**: 398-400.

15 Martin SF, Esser PR, Weber FC, Jakob T, Freudenberg MA, Schmidt M, Goebeler M.

Mechanisms of chemical-induced innate immunity in allergic contact dermatitis. *Allergy* 2011: **66**: 1152-63.

16 Isaksson M, Gruvberger B, Bruze M. Occupational contact allergy and dermatitis from methylisothiazolinone after contact with wallcovering glue and after a chemical burn from a biocide. *Dermatitis* 2004: **15**: 201-5.

17 Enk AH, Katz SI. Early molecular events in the induction phase of contact sensitivity. *Proc Natl Acad Sci U S A* 1992: **89**: 1398-402.

18 Enk AH, Angeloni VL, Udey MC, Katz SI. An essential role for Langerhans cell-derived IL-1 beta in the initiation of primary immune responses in skin. *J Immunol* 1993: **150**: 3698-704.

19 Bergstresser PR. Sensitization and elicitation of inflammation in contact dermatitis. *Immunol Ser* 1989: **46**: 219-45.

20 Watanabe H, Gaide O, Petrilli V, Martinon F, Contassot E, Roques S, Kummer J A, Tschopp J, French L E. Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity. *J Invest Dermatol* 2007: **127**: 1956-63.

21 Bos JD, Meinardi MM. The 500 Dalton rule for the skin penetration of chemical compounds and drugs. *Exp Dermatol* 2000: **9**: 165-9.

22 Divkovic M, Pease CK, Gerberick GF, Basketter DA. Hapten-protein binding: from theory to practical application in the in vitro prediction of skin sensitization. *Contact Dermatitis* 2005: **53**: 189-200.

23 Gerberick F, Aleksic M, Basketter D, Casati S, Karlberg AT, Kern P, Kimber I, Lepoittevin J-P, Natsch A, Ovigne JM, Rovida C, Sakaguchi H, Schultz T. Chemical reactivity measurement and the predicitve identification of skin sensitisers. The report and recommendations of ECVAM Workshop 64. *Altern Lab Anim* 2008: **36**: 215-42.

24 Sargent M (Ed.), Guide to achieving reliable quantitative LC-MS measurements, RSC Analytical Methods Committee, 2013. ISBN 978-0-948926-27-3. Available at: http://www.rsc.org/images/AMC%20LCMS%20Guide_tcm18-240030.pdf (last accessed 7 March 2017).

25 Streilein JW, Bergstresser PR. Langerhans cells: antigen presenting cells of the epidermis. *Immunobiology* 1984: **168**: 285-300.

26 Kimber I, Dearman RJ. Allergic contact dermatitis: the cellular effectors. *Contact Dermatitis* 2002: **46**: 1-5.

27 Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. Skin immune sentinels in health and disease. *Nat Rev Immunol* 2009: **9**: 679-91.

28 Zarember KA, Godowski PJ. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *J Immunol* 2002: **168**: 554-61.

29 Jakob T, Udey MC. Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells in vivo. *J Immunol* 1998: **160**: 4067-73.

30 Kim CH, Broxmeyer HE. Chemokines: signal lamps for trafficking of T and B cells for development and effector function. *J Leukoc Biol* 1999: **65**: 6-15.

31 Villarino AV, Tato CM, Stumhofer JS, Yao Z, Cui YK, Hennighausen L, O'Shea JJ, Hunter CA. Helper T cell IL-2 production is limited by negative feedback and STAT-dependent cytokine signals. *J Exp Med* 2007: **204**: 65-71.

32 Berger A. Th1 and Th2 responses: what are they? *BMJ* 2000: **321**: 424.

33   Jakobson E, Masjedi K, Ahlborg N, Lundeberg L, Karlberg AT, Scheynius A. Cytokine production in nickel-sensitized individuals analysed with enzyme-linked immunospot assay: possible implication for diagnosis. *Br J Dermatol* 2002: **147**: 442-9.

34   Masjedi K, Ahlborg N, Gruvberger B, Bruze M, Karlberg A T. Methylisothiazolinones elicit increased production of both T helper (Th)1- and Th2-like cytokines by peripheral blood mononuclear cells from contact allergic individuals. *Br J Dermatol* 2003: **149**: 1172-82.

35   Gober MD, Gaspari AA. Allergic contact dermatitis. *Curr Dir Autoimmun* 2008: **10**: 1-26.

36   Gober MD, Fishelevich R, Zhao Y, Unutmaz D, Gaspari AA. Human natural killer T cells infiltrate into the skin at elicitation sites of allergic contact dermatitis. *J Invest Dermatol* 2008: **128**: 1460-9.

37   Noh G, Lee JH. Regulatory B cells and allergic diseases. *Allergy Asthma Immunol Res* 2011: **3**: 168-77.

38   Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999: **401**: 708-12.

39   Szepietowski JC, Mckenzie RC, Keohane SG, Walker C, Aldridge RD, Hunter JA. Leukaemia inhibitory factor: induction in the early phase of allergic contact dermatitis. *Contact Dermatitis* 1997: **36**: 21-5.

40   De Groot AC, Herxheimer A. Isothiazolinone preservative: cause of a continuing epidemic of cosmetic dermatitis. *Lancet* 1989: **1**: 314-6.

41   Johansen JD, Veien N, Laurberg G, Avnstorp C, Kaaber K, Andersen KE, Paulsen E, Sommerlund M, Thormann J, Nielsen N H, Vissing S, Kristensen O, Kristensen B, Agner T, Menné T. Decreasing trends in methyldibromo glutaronitrile contact allergy--following regulatory intervention. *Contact Dermatitis* 2008: **59**: 48-51.

42   Mcfadden JP, Ross JS, Jones AB, Rycroft RJ, Smith HR, White IR. Increased rate of patch test reactivity to methyldibromo glutaronitrile. *Contact Dermatitis* 2000: **42**: 54-5.

43   Schnuch A, Lessmann H, Geier J, Uter W. Contact allergy to preservatives. Analysis of IVDK data 1996-2009. *Br J Dermatol* 2011: **164**: 1316-25.

44   Wilkinson JD, Shaw S, Andersen KE, Brandao FM, Bruynzeel DP, Bruze M, Camarasa JM, Diepgen TL, Ducombs G, Frosch PJ, Goossens A, Lachappelle JM, Lahti A, Menné T, Seidenari S, Tosti A, Wahlberg JE. Monitoring levels of preservative sensitivity in Europe. A 10-year overview (1991-2000). *Contact Dermatitis* 2002: **46**: 207-10.

45   Thyssen JP, Engkilde K, Lundov MD, Carlsen BC, Menné T, Johansen JD. Temporal trends of preservative allergy in Denmark (1985-2008). *Contact Dermatitis* 2010: **62**: 102-8.

46   Garcia-Gavin J, Vansina S, Kerre S, Naert A, Goossens A. Methylisothiazolinone, an emerging allergen in cosmetics? *Contact Dermatitis* 2010: **63**: 96-101.

47   Dillarstone A. Cosmetic preservatives. *Contact Dermatitis* 1997: **37**: 190.

48   Menné T, Wahlberg JE. Risk assessment failures of chemicals commonly used in consumer products. *Contact Dermatitis* 2002: **46**: 189-90.

49   Thyssen JP, Menné T, Schnuch A, Uter W, White I, White JM, Johansen JD. Acceptable risk of contact allergy in the general population assessed by CE-DUR--a method to detect and categorize contact allergy epidemics based on patient data. *Regul Toxicol Pharmacol* 2009: **54**: 183-7.

50   Madsen JT, Broesby-Olsen S, Andersen KE. Undisclosed methylisothiazolinone in an ultrasound gel causing occupational allergic contact dermatitis. *Contact Dermatitis* 2014: **71**: 312-3.

51   Dararattanaroj W, Pootongkam S, Rojanawatsirivej N, Wongpiyabovorn J. Patterns and risk factors of causative contact allergens in Thai adult patients with contact dermatitis at King Chulalongkorn Memorial Hospital. *Asian Pac J Allergy Immunol* 2016.

52   Castanedo-Tardana MP, Zug KA. Methylisothiazolinone. *Dermatitis* 2013: **24**: 2-6.

53   Cheng S, Leow YH, Goh CL, Goon A. Contact sensitivity to preservatives in Singapore: frequency of sensitization to 11 common preservatives 2006-2011. *Dermatitis* 2014: **25**: 77-82.

54   Dekoven JG, Warshaw EM, Belsito DV, Sasseville D, Maibach HI, Taylor JS, Marks JG, Fowler JF Jr, Mathias CG, Deleo VA, Pratt MD, Zirwas MJ, Zug KA. North American Contact Dermatitis Group Patch Test Results 2013-2014. *Dermatitis* 2017: **28**: 33-46.

55   Toholka R, Wang YS, Tate B, Tam M, Cahill J, Palmer A, Nixon R. The first Australian Baseline Series: Recommendations for patch testing in suspected contact dermatitis. *Australas J Dermatol* 2015: **56**: 107-15.

56   Warshaw EM, Maibach HI, Taylor JS, Sasseville D, Dekoven JG, Zirwas MJ, Fransway AF, Mathias CG, Zug KA, Deleo VA, Fowler JF Jr, Marks JG, Pratt MD, Storrs FJ, Belsito DV. North American contact dermatitis group patch test results: 2011-2012. *Dermatitis* 2015: **26**: 49-59.

57   Wright AM, Cahill JL. Airborne exposure to methylisothiazolinone in paint causing allergic contact dermatitis: An Australian perspective. *Australas J Dermatol* 2015.

58   The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCCNFP). Opinion Concerning Methylisothiazolinone (Colipa No. P94). Adopted on 18 March 2003, SCCNFP/0625/02. Available at: http://ec.europa.eu/health/archive/ph_risk/committees/sccp/documents/out_201.pdf (Accessed on 6 March 2017).

59   The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers (SCNNFP). Opinion Concerning Methylisothiazolinone (Colipa No. P94). Adopted on 23 April 2004, SCCNFP/0805/04. Available at: http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out270_en.pdf (Accessed on 1 March 2017). .

60   Basketter D, Wright Z, Walters T, Boman A, Lidén C. Biocides: Characterization of the allergenic hazard of methylisothiazolinone. *Cutan. Ocul. Toxicol.* 2003;22:187–199. .

61   Mose AP, Lundov MD, Zachariae C, Menné T, Veien NK, Laurberg G, Kaaber K, Avnstorp C, Andersen KE, Paulsen E, Mortz CG, Sommerlund M, Danielsen A, Thormann J, Kristensen O, Kristensen B, Andersen BL, Vissing S, Nielsen NH, Johansen JD. Occupational contact dermatitis in painters: an analysis of patch test data from the Danish Contact Dermatitis Group. *Contact Dermatitis* 2012: **67**: 293-7.

62   Schwensen JF, Menné T, Andersen KE, Sommerlund M, Johansen JD. Occupations at risk of developing contact allergy to isothiazolinones in Danish contact dermatitis patients: results from a Danish multicentre study (2009-2012). *Contact Dermatitis* 2014: **71**: 295-302.

63   Scientific Committee on Consumer Safety (SCCS). Opinion on Methylisothiazolinone (P94) – Submission II (sensitisation only). Adopted on 27 March 2014, SCCS/1521/13. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_145.pdf (last accessed 1 March 2017).

64   Scientific Committee on Consumer Safety (SCCS). Opinion on Methylisothiazolinone (P94) – Submission III. Adopted on 15 December 2015, SCCS/1557/15. Available at:

http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_178.pdf (last accessed 1 March 2017).

65   Yazar K, Lundov MD, Faurschou A, Matura M, Boman A, Johansen JD, Lidén C. Methylisothiazolinone in rinse-off products causes allergic contact dermatitis: a repeated open-application study. *Br J Dermatol* 2015: **173**: 115-22.

66   European Commission (EC). Official Journal of the European Union. Regulations. Commission Regulation (EU) 2016/1198 of 22 July 2016 amending Annex V to Regulation (EC) No 1223/2009 of the European Parliament and of the council on cosmetic products. Published 23 July 2016. Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32016R1198&from=EN (last accessed 1 March 2017).

67   European Commission (EC). Directorate General for Internal Market I, Entrepreneurship and SMEs. Public Consultation on Methylisothiazolinone (MI) in the Framework of Regulation (EC) No. 1223/209 – Rinse-Off Cosmetic Products. Published 01/04/2016. Available At: http://ec.europa.eu/growth/tools-databases/newsroom/cf/itemdetail.cfm?item_id=8740 (last accessed 1 March 2017).

68   European Commission. Directorate General for Internal Market, Industry, Entrepreneurship and SMEs. Summary Report from the public consultation on Methylisothiazolinone (rinse-off cosmetic products) in the framework of Regulation (EC) No. 1223/2009 of the European Parliament and of the Council on cosmetic products. Published 26 September 2016. Available at: http://ec.europa.eu/DocsRoom/documents/18861/attachments/1/translations/en/renditions/pdf (last accessed 28 February 2018)

69   Lundov MD, Opstrup MS, Johansen JD. Methylisothiazolinone contact allergy--growing epidemic. *Contact Dermatitis* 2013: **69**: 271-5.

70   Yazar K, Johnsson S, Lind M L, Boman A, Lidén C. Preservatives and fragrances in selected consumer-available cosmetics and detergents. *Contact Dermatitis* 2011: **64**: 265-72.

71   Uter W, Yazar K, Kratz E M, Mildau G, Lidén C. Coupled exposure to ingredients of cosmetic products: II. Preservatives. *Contact Dermatitis* 2014: **70**: 219-26.

72   Magnano M, Silvani S, Vincenzi C, Nino M, Tosti A. Contact allergens and irritants in household washing and cleaning products. *Contact Dermatitis* 2009: **61**: 337-41.

73   Schnuch A, Mildau G, Kratz E M, Uter W. Risk of sensitization to preservatives estimated on the basis of patch test data and exposure, according to a sample of 3541 leave-on products. *Contact Dermatitis* 2011: **65**: 167-74.

74   Garcia-Hidalgo E, Sottas V, Von Goetz N, Hauri U, Bogdal C, Hungerbuhler K. Occurrence and concentrations of isothiazolinones in detergents and cosmetics in Switzerland. *Contact Dermatitis* 2017: **76**: 96-106.

75   Gallo R, Signori A, Gervasio S, Riva S, Parodi A. Methylisothiazolinone contact allergy - are rinse-off cosmetics and household products relevant sources of exposure? *Contact Dermatitis* 2016: **75**: 319-321.

76   Isaksson M, Persson L. 'Mislabelled' make-up remover wet wipes as a cause of severe, recalcitrant facial eczema. *Contact Dermatitis* 2015: **73**: 56-9.

77   Vanneste L, Persson L, Zimerson E, Bruze M, Luyckx R, Goossens A. Allergic contact dermatitis caused by methylisothiazolinone from different sources, including 'mislabelled' household wet wipes. *Contact Dermatitis* 2013: **69**: 311-2.

78   Madsen JT, Andersen KE, Nielsen DT, Hvid L, El-Houri RB, Christensen LP. Undisclosed

presence of methylisothiazolinone in '100% natural' Konjac(R) sponge. *Contact Dermatitis* 2016: **75**: 308-309.

79 Yu J, Treat J, Chaney K, Brod B. Potential Allergens in Disposable Diaper Wipes, Topical Diaper Preparations, and Disposable Diapers: Under-recognized Etiology of Pediatric Perineal Dermatitis. *Dermatitis* 2016: **27**: 110-8.

80 Aerts O, Meert H, Goossens A, Janssens S, Lambert J, Apers S. Methylisothiazolinone in selected consumer products in Belgium: Adding fuel to the fire? *Contact Dermatitis* 2015: **73**: 142-9.

81 Gruvberger B, Bruze M, Almgren G. Occupational dermatoses in a plant producing binders for paints and glues. *Contact Dermatitis* 1998: **38**: 71-7.

82 Reinhard E, Waeber R, Niederer M, Maurer T, Maly P, Scherer S. Preservation of products with MCI/MI in Switzerland. *Contact Dermatitis* 2001: **45**: 257-64.

83 Hardcastle N J, Gawkrodger D J. Occupational contact dermatitis to 1,2-benzisothiazolin-3-one and 5-chloro-2-methylisothiazolin-3-one/2-methylisothiazolin-3-one in paint manufacturers. *Contact Dermatitis* 2005: **53**: 115-6.

84 Thyssen JP, Sederberg-Olsen N, Thomsen JF, Menné T. Contact dermatitis from methylisothiazolinone in a paint factory. *Contact Dermatitis* 2006: **54**: 322-4.

85 Lundov MD, Kolarik B, Bossi R, Gunnarsen L, Johansen JD. Emission of isothiazolinones from water-based paints. *Environ Sci Technol* 2014: **48**: 6989-94.

86 Friis U F, Menné T, Flyvholm MA, Bonde JP, Lepoittevin J-P, Le Coz CJ, Johansen JD. Isothiazolinones in commercial products at Danish workplaces. *Contact Dermatitis* 2014: **71**: 65-74.

87 Schwensen JF, Menné T, Friis UF, Johansen JD. Undisclosed methylisothiazolinone in wet wipes for occupational use causing occupational allergic contact dermatitis in a nurse. *Contact Dermatitis* 2015: **73**: 182-4.

88 Bennike NH, Johansen JD, Zachariae C. Please, label the label; a case report of occupational allergic contact dermatitis caused by methylisothiazolinone in adhesive labels. *Contact Dermatitis* 2016: **75**: 314-315.

89 European Chemicals Agency (ECHA). Guidance on labelling and packaging in accordance with regulation (EC) No. 1272/2008, Reference ECHA-11-G-04-EN 2011 April. Available at: https://echa.europa.eu/documents/10162/13562/clp_labelling_en.pdf (last accessed 1 March 2017).

90 European Chemicals Agency (ECHA). Committee for risk assessment, RAC. Opinion, proposing harmonised classification and labelling at EU level of 2-methylisothiazol-3(2H)-one (ISO), Adopted 10 March 2016. Available at: http://echa.europa.eu/documents/10162/b32e77aa-b720-4f66-ad16-a413dadf1631 (last accessed 6 March 2017).

91 Kawakami T, Isama K, Ikarashi Y. Analysis of isothiazolinone preservatives in polyvinyl alcohol cooling towels used in Japan. *J Environ Sci Health A Tox Hazard Subst Environ Eng* 2014: **49**: 1209-17.

92 Kaae J, Menné T, Thyssen JP. Presumed primary contact sensitization to methylisothiazolinone from paint: a chemical that became airborne. *Contact Dermatitis* 2012: **66**: 341-2.

93 Lundov MD, Friis UF, Menné T, Johansen JD. Methylisothiazolinone in paint forces a patient out of her apartment. *Contact Dermatitis* 2013: **69**: 252-3.

94   Alwan W, White IR, Banerjee P. Presumed airborne contact allergy to methylisothiazolinone causing acute severe facial dermatitis and respiratory difficulty. *Contact Dermatitis* 2014: **70**: 320-1.

95   Breuer K, Uter W, Geier J. Epidemiological data on airborne contact dermatitis - results of the IVDK. *Contact Dermatitis* 2015: **73**: 239-47.

96   Flyvholm MA. Preservatives in registered chemical products. *Contact Dermatitis* 2005: **53**: 27-32.

97   Fischer R, Hurni H. On Benzisothiazolones: A Series with a Wide Range of Bacteriostatic and Fungistatic Activity. *Arzneimittelforschung* 1964: **14**: 1301-6.

98   Bruze M, Uter W, Gonçalo M, Lepoittevin J-P, Diepgen T, Orton D. Incompetence and failure to regulate methylisothiazolinone. *Contact Dermatitis* 2015: **72**: 353-4.

99   Aalto-Korte K, Ackermann L, Henriks-Eckerman ML, Valimaa J, Reinikka-Railo H, Leppanen E, Jolanki R. 1,2-benzisothiazolin-3-one in disposable polyvinyl chloride gloves for medical use. *Contact Dermatitis* 2007: **57**: 365-70.

100 Taran JM, Delaney TA. Allergic contact dermatitis to 1,2-benzisothiazolin-3-one in the carpet industry. *Australas J Dermatol* 1997: **38**: 42-3.

101 Dias M, Lamarao P, Vale T. Occupational contact allergy to 1,2-benzisothiazolin-3-one in the manufacture of air fresheners. *Contact Dermatitis* 1992: **27**: 205-7.

102 Freeman S. Allergic contact dermatitis due to 1,2-benzisothiazolin-3-one in gum arabic. *Contact Dermatitis* 1984: **11**: 146-9.

103 Alomar A. Contact dermatitis from benzisothiazolone in cutting oils. *Contact Dermatitis* 1981: **7**: 155-6.

104 Roberts DL, Messenger AG, Summerly R. Occupational dermatitis due to 1,2-benzisothiazolin-3-one in the pottery industry. *Contact Dermatitis* 1981: **7**: 145-7.

105 Damstra RJ, Van Vlotten WA, Van Ginkel CJ. Allergic contact dermatitis from the preservative 1,2-benzisothiazolin-3-one (1,2-BIT; Proxel): a case report, its prevalence in those occupationally at risk and in the general dermatological population, and its relationship to allergy to its analogue Kathon CG. *Contact Dermatitis* 1992: **27**: 105-9.

106 Geier J, Lessmann H, Schnuch A, Uter W. Concomitant reactivity to methylisothiazolinone, benzisothiazolinone, and octylisothiazolinone. International Network of Departments of Dermatology data, 2009-2013. *Contact Dermatitis* 2015: **72**: 337-9.

107 Sanz-Gallén P, Planas J, Martinez P, Giménez-Arnau JM. Allergic contact dermatitis due to 1,2-benzisothiazolin-3-one in paint manufacture. *Contact Dermatitis* 1992: **27**: 271-2.

108 Fischer T, Bohlin S, Edling C, Rystedt I, Wieslander G. Skin disease and contact sensitivity in house painters using water-based paints, glues and putties. *Contact Dermatitis* 1995: **32**: 39-45.

109 Meding B, Ahman M, Karlberg AT. Skin symptoms and contact allergy in woodwork teachers. *Contact Dermatitis* 1996: **34**: 185-90.

110 Meysman T, Goossens A. Occupational allergic contact dermatitis caused by benzisothiazolinone in printing ink and soap. *Contact Dermatitis* 2017: **76**: 51-53.

111 Aerts O, Meert H, Romaen E, Leysen J, Matthieu L, Apers S, Lambert J, Goossens A. Octylisothiazolinone, an additional cause of allergic contact dermatitis caused by leather: case series and potential implications for the study of cross-reactivity with methylisothiazolinone. *Contact Dermatitis* 2016: **75**: 276-284.

112 Mose AP, Frost S, Ohlund U, Andersen KE. Allergic contact dermatitis from

octylisothiazolinone. *Contact Dermatitis* 2013: **69**: 49-52.

113 Bruze M, Gruvberger B, Persson K. Contact allergy to a contaminant in Kathon CG in the guinea pig. *Derm Beruf Umwelt* 1987: **35**: 165-8.

114 Geier J, Schnuch A. No cross-sensitization between MCI/MI, benzisothiazolinone and octylisothiazolinone. *Contact Dermatitis* 1996: **34**: 148-9.

115 Isaksson M, Bruze M, Gruvberger B. Cross-reactivity between methylchloroisothiazolinone/methylisothiazolinone, methylisothiazolinone, and other isothiazolinones in workers at a plant producing binders for paints and glues. *Contact Dermatitis* 2008: **58**: 60-2.

116 Johansen JD, Aalto-Korte K, Agner T, Andersen KE, Bircher A, Bruze M, Cannavo A, Giménez-Arnau A, Gonçalo M, Goossens A, John SM, Lidén C, Lindberg M, Mahler V, Matura M, Rustemeyer T, Serup J, Spiewak R, Thyssen JP, Vigan M, White IR, Wilkinson M, Uter W. European Society of Contact Dermatitis guideline for diagnostic patch testing - recommendations on best practice. *Contact Dermatitis* 2015: **73**: 195-221.

117 Scientific Committee on Consumer Safety. Opinion on Benzisothiazolinone: European Commission Health & Consumers. Colipa no P96. Published 26 June 2012, SCCS/1482/12. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_099.pdf (last accessed 1 March 2017).

118 European Commission. Reports of the Scientific Committee on Cosmetology (second series). EUR 8634. Published 1983. Available at: https://ec.europa.eu/health/sites/health/files/scientific_committees/consumer_safety/docs/scc_o_2.pdf (last accessed 6 March 2017)

119 Bruze M, Gruvberger B, Agrup G. Sensitization studies in the guinea pig with the active ingredients of Euxyl K 400. *Contact Dermatitis* 1988: **18**: 37-9.

120 Andersen, F. Final report on the safety assessment of methyldibromo glutaronitrile. *J. Am. Cell Toxicol.* 1996;15:140–165.

121 Schwensen JF, Thyssen JP. Contact Allergy to Preservatives–Is the European Commission a Commendable Risk Manager? *Cosmetics* 2016;3:29.

122 Wahlkvist H, Boman A, Montelius J, Wahlberg J E. Sensitizing potential in mice, guinea pig and man of the preservative Euxyl K 400 and its ingredient methyldibromo glutaronitrile. *Contact Dermatitis* 1999: **41**: 330-8.

123 De Groot AC, De Cock PA, Coenraads PJ, Van Ginkel CJ, Jagtman BA, Van Joost T, Joost Van Der Kley AM, Meinardi MM, Smeenk G, Van Der Valk PG, Van Der Walle HB, Weyland JW. Methyldibromoglutaronitrile is an important contact allergen in The Netherlands. *Contact Dermatitis* 1996: **34**: 118-20.

124 Van Ginkel CJ, Rundervoort GJ. Increasing incidence of contact allergy to the new preservative 1,2-dibromo-2,4-dicyanobutane (methyldibromoglutaronitrile). *Br J Dermatol* 1995: **132**: 918-20.

125 Vigan M, Brechat P. Sensitization to Euxyl K400: A prospective study in 1217 patients. *Eur. J. Dermatol.* 1996;6:175–177.

126 Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP). Opinion Concerning Methyldibromo Glutaronitrile (Colipa No. P77), Adopted on 4 June 2002, SCCNFP/0585/02. Available at: http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out169_en.pdf (last

accessed on 1 March 2017).

127 Johansen JD, Veien NK, Laurberg G, Kaaber K, Thormann J, Lauritzen M, Avnstorp C. Contact allergy to methyldibromo glutaronitrile--data from a 'front line' network. *Contact Dermatitis* 2005: **52**: 138-41.

128 Zachariae C, Johansen JD, Rastogi SC, Menné T. Allergic contact dermatitis from methyldibromo glutaronitrile--clinical cases from 2003. *Contact Dermatitis* 2005: **52**: 6-8.

129 Scientific Committee on Consumer Products (SCCP). Opinion on Methyldibromo Glutaronitrile (Sensitization only) (SCCP/0863/05); COLIPA No. P77. 15 March 2005. Available online: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_060.pdf (last accessed on 1 March 2017).

130 Jong CT, Statham BN, Green CM, King CM, Gawkrodger DJ, Sansom JE, English JS, Wilkinson SM, Ormerod AD, Chowdhury MM. Contact sensitivity to preservatives in the UK, 2004-2005: results of multicentre study. *Contact Dermatitis* 2007: **57**: 165-8.

131 European Commission. Council Directive of 27 July 1976 on the Approximation of the Laws of the Member States Relating to Cosmetic Products (1976L0768-EN-24.04.2008-021.002-3). Available at: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:31976L0768&from=EN (accessed on 6 March 2017).

132 The SCCS Notes of Guidance for the Testing of Cosmetic Ingredient and Their Safety Evaluation, 9th Revision. SCCS/1564/15. Revised 25 April 2016. Available online: http://ec.europa.eu/health/scientific_committees/consumer_safety/docs/sccs_o_190.pdf (accessed on 6 March 2017).

133 European Commission. Regulation (EC) No. 1223/2009 of the European Parliament and of the Council of 30 November 2009 on Cosmetic Products. Available online: http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32009R1223&from=EN (last accessed on 6 March 2017)

134 Scientific Committee on Consumer Safety (SCCS). Health and Food Safety. Scientific Committees. European Commission. Available at: https://ec.europa.eu/health/scientific_committees/consumer_safety_en (accessed on 6 March 2017).

135 Scientific Committee on Consumer Safety (SCCS). SCCS – Members. Available at: http://ec.europa.eu/health/scientific_committees/consumer_safety/members_committee/index_en.htm (last accessed 6 March 2017).

136 Uter W, Schnuch A, Wilkinson M, Dugonik A, Dugonik B, Ganslandt T. Registries in Clinical Epidemiology: the European Surveillance System on Contact Allergies (ESSCA). *Methods Inf Med* 2016: **55**: 193-9.

137 Uter W, Rustemeyer T, Wilkinson M, Johansen JD. Quality in epidemiological surveillance of contact allergy. *Contact Dermatitis* 2016: **74**: 175-80.

138 Steiling, W. Safety evaluation of cosmetic ingredients regarding their skin sensitization potential. Cosmetics. 2016;3:14.

139 Kimber I, Dearman RJ, Basketter DA, Ryan CA, Gerberick GF. The local lymph node assay: past, present and future. *Contact Dermatitis* 2002: **47**: 315-28.

140 Uter W, Schnuch A, Gefeller O. Guidelines for the descriptive presentation and statistical analysis of contact allergy data. *Contact Dermatitis* 2004: **51**: 47-56.

141 Friis UF, Menné T, Flyvholm MA, Bonde JP, Johansen JD. Difficulties in using Material Safety

Data Sheets to analyse occupational exposures to contact allergens. *Contact Dermatitis* 2015: **72**: 147-53.

142 Schwensen JF, Friis UF, Menné T, Flyvholm MA, Johansen JD. Contact allergy to preservatives in patients with occupational contact dermatitis and exposure analysis of preservatives in registered chemical products for occupational use. *Int Arch Occup Environ Health* 2017.

143 Warbrick EV, Dearman RJ, Basketter DA, Kimber I. Influence of application vehicle on skin sensitization to methylchloroisothiazolinone/methylisothiazolinone: an analysis using the local lymph node assay. *Contact Dermatitis* 1999: **41**: 325-9.

144 Roberts DW. Methylisothiazolinone is categorised as a strong sensitiser in the Murine Local Lymph Node Assay. *Contact Dermatitis* 2013: **69**: 261-2.

145 Gerberick GF, Ryan CA, Dearman RJ, Kimber I. Local lymph node assay (LLNA) for detection of sensitization capacity of chemicals. *Methods* 2007: **41**: 54-60.

146 Pontén A, Bruze M, Engfeldt M, Hauksson I, Isaksson M. Concomitant contact allergies to formaldehyde, methylchloroisothiazolinone/methylisothiazolinone, methylisothiazolinone, and fragrance mixes I and II. *Contact Dermatitis* 2016: **75**: 285-289.

147 Linauskiene K, Malinauskiene L, Blaziene A. Time trends of contact allergy to the European baseline series in Lithuania. *Contact Dermatitis* 2016.

148 Beliauskiene A, Valiukeviciene S, Uter W, Schnuch A. The European baseline series in Lithuania: results of patch testing in consecutive adult patients. *J Eur Acad Dermatol Venereol* 2011: **25**: 59-63.

149 Giménez-Arnau AM, Deza G, Bauer A, Johnston GA, Mahler V, Schuttelaar M L, Sanchez-Perez J, Silvestre J F, Wilkinson M, Uter W. Contact allergy to preservatives: ESSCA* results with the baseline series, 2009-2012. *J Eur Acad Dermatol Venereol* 2016.

150 Amaro C, Cravo M, Fernandes C, Santos R, Gonçalo M. Undisclosed methyldibromo glutaronitrile causing allergic contact dermatitis in a NSAID cream. *Contact Dermatitis* 2012: **67**: 173-4.

151 Lundov MD, Mosbech H, Thyssen JP, Menné T, Zachariae C. Two cases of airborne allergic contact dermatitis caused by methylisothiazolinone in paint. *Contact Dermatitis* 2011: **65**: 176-9.

152 Aerts O, Cattaert N, Lambert J, Goossens A. Airborne and systemic dermatitis, mimicking atopic dermatitis, caused by methylisothiazolinone in a young child. *Contact Dermatitis* 2013: **68**: 250-1.

153 Kaur-Knudsen D, Menné T, Christina Carlsen B. Systemic allergic dermatitis following airborne exposure to 1,2-benzisothiazolin-3-one. *Contact Dermatitis* 2012: **67**: 310-2.

154 Tokunaga M, Fujii H, Okada K, Kagemoto Y, Nomura T, Tanioka M, Matsumura Y, Miyachi Y. Occupational airborne contact dermatitis by isothiazolinones contained in wall paint products. *Allergol Int* 2013: **62**: 395-7.

155 Bregnbak D, Johansen JD. Airborne sensitization to isothiazolinones observed in a 3-month-old boy. *Contact Dermatitis* 2013: **69**: 55-6.

156 Bregnbak D, Lundov MD, Zachariae C, Menné T, Johansen JD. Five cases of severe chronic dermatitis caused by isothiazolinones. *Contact Dermatitis* 2013: **69**: 57-9.

157 Opstrup MS, Johansen JD, Zachariae C, Garvey LH. Contact allergy to chlorhexidine in a tertiary dermatology clinic in Denmark. *Contact Dermatitis* 2016: **74**: 29-36.

158 Devos FC, Pollaris L, Van Den Broucke S, Seys S, Goossens A, Nemery B, Hoet PH, Vanoirbeek JA. Methylisothiazolinone: dermal and respiratory immune responses in mice. *Toxicol Lett*

2015: **235**: 179-88.

159 Debeuckelaere C, Moussallieh FM, Elbayed K, Namer IJ, Berl V, Giménez-Arnau E, Lepoittevin J-P. In situ chemical behaviour of methylisothiazolinone (MI) and methylchloroisothiazolinone (MCI) in reconstructed human epidermis: a new approach to the cross-reactivity issue. *Contact Dermatitis* 2016: **74**: 159-67.

160 Venables ZC, Bourke JF, Buckley DA, Campbell F, Chowdhury MM, Abdul-Ghaffar S, Green C, Holden C R, Mcfadden J, Orton D, Sabroe R A, Sansom J, Stone NM, Wakelin SH, Wilkinson SM, Johnston GA. Has the epidemic of allergic contact dermatitis due to Methylisothiazolinone reached its peak? *Br J Dermatol* 2016.

161 Heisterberg MV, Menné T, Johansen JD. Fragrance allergy and quality of life - a case-control study. *Contact Dermatitis* 2014: **70**: 81-9.

162 Lau MY, Matheson MC, Burgess JA, Dharmage S C, Nixon R. Disease severity and quality of life in a follow-up study of patients with occupational contact dermatitis. *Contact Dermatitis* 2011: **65**: 138-45.

163 Martin-Gorgojo A, Curto-Barredo L, Rovira-Lopez R, Pujol R M, Giménez-Arnau A. Is methylisothiazolinone contact allergy a risk factor for polysensitization? *Contact Dermatitis* 2015: **72**: 400-2.

164 Cosmetics Europe. Recommendation on MI. Published 12 December 2013. Available at: https://www.cosmeticseurope.eu/files/3614/7634/5470/Recommendation_on_MIT.pdf (accessed 1 March 2017).

165 Schwensen JF, Bregnbak D, Johansen JD. Recent trends in epidemiology, sensitization and legal requirements of selected relevant contact allergens. *Expert Rev Clin Immunol* 2016: **12**: 289-300.